Role of type I interferons in *Streptococcus pneumoniae* pneumonia

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Zusammenfassung

Streptococcus pneumoniae ist die häufigste Ursache für ambulant erworbene Pneumonien weltweit. Daher müssen die Wirts-Pathogen-Interaktionen erforscht werden, um neue Therapiestrategien zu entwickeln. In dieser Studie habe ich 1. den Typ I Interferon (IFN)-stimulierenden Signalweg des angeborenen Immunsystems in Pneumokokken-infizierten Wirtszellen sowie 2. dessen Bedeutung in der Pneumokokkenpneumonie untersucht.

Humane und murine Makrophagen, aber nicht alveolare Epithelzellen, produzierten Typ I IFNs nach Infektion mit *S. pneumoniae*. Dieses war abhängig vom Virulenzfaktor Pneumolysin und erforderte sowohl die Phagozytose der Bakterien als auch die Ansäuerung der Endosomen. Die Induktion der Typ I IFNs wird durch einen zytosolischen Signalweg vermittelt, welcher wahrscheinlich DNA erkennt und sowohl das Adapterprotein STING als auch den Transkriptionsfaktor IRF3 aktiviert.

Typ I IFNs, welche von infizierten Makrophagen gebildet wurden, regulierten die Expression von IFN-stimulierten Genen (ISGs) und Chemokinen in Makrophagen und co-kultivierten alveolaren Epithelzellen *in vitro* und in Mauslungen *in vivo*. In einem murinen Pneumoniemodell hatten die Typ I IFNs jedoch einen negativen Effekt für den Wirt. Mäuse mit einem Defekt im Typ I IFN-Rezeptor oder mit einem Knockout im Typ I und Typ II IFN-Rezeptor hatten eine signifikant geringere Bakterienlast in der Lunge und eine verminderte Reduktion der Körpertemperatur und des Körpergewichtes als wild-typ Mäuse. Diese Effekte waren nicht durch Änderungen in der Zellrekrutierung oder durch Änderungen der Zytokin-/Chemokinexpression erklärbar.

Zusammenfassend lässt sich feststellen, dass Typ I IFNs durch Pneumokokken induziert werden, aber dass sie trotz einiger positiver Effekte auf die Expression von ISGs einen negativen Gesamteffekt in einem murinen Pneumoniemodell aufweisen. Ein detailliertes Verständnis der Typ I IFN-Antwort während der Pneumokokkeninfektion kann die Entwicklung neuer Therapiestrategien unterstützen.

Schlagwörter: *Streptococcus pneumoniae*, Typ I Interferone, DNA, Pneumonie

Summary

Streptococcus pneumoniae is the leading cause of community-acquired pneumonia world-wide. A detailed understanding of the host-pathogen interactions is required in order to foster the development of new therapeutic strategies. Here, I (I) characterized an innate immune recognition pathway that senses pneumococcal infection and triggers the production of type I interferons (IFNs), and (II) examined the role of type I IFNs in pneumococcal pneumonia in mice.

Human and murine macrophages, but not alveolar epithelial cells, produced type I IFNs after infection with *S. pneumoniae*. This induction was dependent on the virulence factor pneumolysin, the phagocytosis of the bacteria, and the acidification of the endosome. Moreover, it appeared to be mediated by a cytosolic DNA-sensing pathway involving the adaptor molecule STING and the transcription factor IRF3.

Type I IFNs produced by *S. pneumoniae*-infected macrophages positively regulated the expression of IFN-stimulated genes (ISGs) and chemokines in macrophages and co-cultured alveolar epithelial cells *in vitro* and in mouse lungs *in vivo*. However, in a murine model of pneumococcal pneumonia, type I IFN signaling was detrimental to the host defense. Mice deficient in the type I IFN signaling or double deficient in type I and type II IFN signaling had a significantly reduced bacterial load in the lung and a diminished reduction of body temperature and body weight compared to wild-type mice. The decreased susceptibility of the knockout mice was unlikely to be attributable to alterations in cell recruitment or cytokine/chemokine production.

In conclusion, type I IFNs are induced during pneumococcal infection. However, despite their positive effects on the expression of some ISGs and chemokines, they negatively affect the outcome of pneumococcal pneumonia in an *in vivo* mouse model. Targeting the type I IFN system could potentially be an effective way in enhancing the immune response in patients with *S. pneumoniae* pneumonia.

Keywords: *Streptococcus pneumoniae*, type I interferons, DNA, pneumonia

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Abbreviations

Tab. 1: List of abbreviations

AIM2	absent in melanoma 2
APC	antigen-presenting cell
ASC	apoptosis-associated speck-like protein containing CARD
BAL	broncho-alveolar lavage
BMM	bone marrov-derived macrophage
B6	C57BL/6
CBP	CREB (cAMP response element-binding protein) binding protein
CD	cluster of differentiation
CFU	colony-forming unit
CpG	short for: cytidine-phosphate-guanosine DNA sequence
Cps	pneumococcal capsule
CXCL5	chemokine (C-X-C motif) ligand 5
cyclic di-AMP/GMP	3',5'-cyclic diadenylic/diguanylic acid
C1q	complement component 1, q subcomponent
DAI	DNA-dependent activator of IFN regulatory factors
DAMP	danger-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cell
DDX1, 21, 41, 58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 1, 21, 41, 58
DHX9, 36	DEAH (Asp-Glu-Ala-His) box polypeptide 9, 36
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig.	Figure
FISH	fluorescence in situ hybridization
F. tularensis	*Francisella tularensis*
GAF	gamma-interferon activation factor

GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GAS	gamma interferon activation site
GM-CSF	granulocyte macrophage colony-stimulating factor
GpIIA-PLA2	Group IIA phospholipase A2
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIN200	hematopoietic interferon-inducible nuclear antigens with 200 amino acid repeats
HIV	human immunodeficiency virus
IFI16	interferon, gamma-inducible protein 16
IFN	interferon
IFNAR	interferon α/β receptor
IFNGR	interferon γ receptor
IFNα/β	type I IFN
IL	interleukin
IgG, IgM	immunoglobulin G, immunoglobulin M
iNKT cell	invariant natural killer T cell
IPD	invasive pneumococcal disease
IRAK	IL-1R-associated kinase
IRF	interferon-regulatory factor
IRG	immunity-related GTPase
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
ISRE	interferon-stimulated response element
JAK	janus kinase
KC	CXCL1 chemokine
L. pneumophila	*Legionella pneumophila*
L. monocytogenes	*Listeria monocytogenes*
LPS	lipopolysaccharide
LRRFIP-1	leucine rich repeat (in FLII) interacting protein 1
LRT	lower respiratory tract
Mal	MyD88 adaptor-like
MALP2	macrophage-activating lipopeptide
MAP kinases	mitogen-activated protein kinases
MAVS	mitochondrial antiviral-signaling protein
MBL	mannan-binding lectin

MCP-1	monocytes chemotactic protein-1
MCS-F	macrophage colony-stimulating factor
MDA5	melanoma differentiation-associated gene 5
mDC	myeloid dendritic cell
MDP	muramyl dipeptide
MHC	major histocompatibility complex
MIP-2	macrophage inflammatory protein 2
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger RNA
Mx protein	myxovirus (influenza virus) resistance protein
MyD88	myeloid differentiation primary response gene 88, which also mediates IL-1 receptor and IL-18 receptor signaling
M. tuberculosis	*Mycobacterium tuberculosis*
NET	neutrophil extracellular trap
NF-κB	nuclear factor-κB
NLR	NOD-like receptor
NLRP3	NOD-like receptor family, pyrin domain containing 3
NOD	nucleotide-binding oligomerization domain-containing protein
n.s.	not significant
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
pDC	plasmacytoid dendritic cell
Pen/Strep	Penicillin/Streptomycin
p.i.	post infection
PLY	pneumolysin
PMN	polymorphonuclear leukocytes
poly dA:dT	poly(deoxyadenylic:deoxythymidylic) acid
poly dG:dC	poly(deoxyguanylic:deoxycytidylic) acid
poly I:C	poly(inosinic:cytidylic) acid
PRR	pattern recognition receptor
PYHIN	pyrin and HIN200 domain–containing proteins
p300	E1A binding protein p300
PKR	double-stranded RNA-dependent protein kinase
RANTES	regulated upon activation, normal T cell expressed and

	presumably secreted
RIG-I	retinoic acid-inducible gene I
RIP2	receptor-interacting protein 2
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
siRNA	small interfering RNA
ssRNA	single-stranded RNA
STAT	signal transducer and activator of transcription
STING	stimulator of IFN genes
S. pneumoniae	*Streptococcus pneumoniae*
S. typhimurium	*Salmonella typhimurium*
Tab.	Table
TAMRA	5-carboxytetramethylrhodamine
THY	Todd-Hewitt broth + yeast extract
TLR	toll-like receptor
TNFα	tumor necrosis factor α
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFNβ
URT	upper respiratory tract
ZBP-1	Z-DNA-binding protein 1
6-FAM	6-Carboxyfluorescein

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1. Introduction

1.1 *Streptococcus pneumoniae*

1.1.1 Biology

Streptococcus pneumoniae, also known as "the pneumococcus", is a gram-positive bacterium that frequently colonizes the upper respiratory tract (URT) of humans but can also cause invasive pneumococcal disease (IPD) (Lynch and Zhanel, 2010). Its genome contains approximately 2.1 million base pairs that encode an approximate number of around 1880 genes. These include 1553 genes essential for the viability, 154 genes required for virulence, and 176 genes that can actively maintain a non-invasive phenotype (van der Poll and Opal, 2009). Pneumococci are surrounded by a polysaccharide capsule that determines their serotype. To this date, more than 90 different serotypes of *S. pneumoniae* are known (Kadioglu, et al., 2008).

On their surface, pneumococci express a diverse array of transporters that mediate the in- and export of substrates and proteins, but that also play a role in acquiring genetic competence and resistance to antibiotics (van der Poll and Opal, 2009). Other surface proteins are involved in the adhesion of *S. pneumoniae* to epithelial surfaces, tissue invasion, and interference with the immune response of the host (Beiter, et al., 2006; Mitchell and Mitchell, 2010).

The pneumococcal capsule itself is an important virulence factor. It is covalently attached to peptidoglycans of the outer cell wall of *S. pneumoniae*. It has a thickness of approximately 200-400 nm. The surface of the capsule carries a high negative charge that electrostatically repulses the sialic acid moieties in the host mucus and prevents the mechanical clearance from the nasopharynx (Kadioglu, et al., 2008; Nelson, et al., 2007). The capsule also protects *S. pneumoniae* against the immune response of the host by impairing pneumococcal opsonization with complement factors, immunoglobulins, and the C-reactive protein, as well as by inhibiting phagocytosis and killing by neutrophils (Hyams, et al., 2010). During the early phase of colonization, pneumococci tend to express thin capsules in order to permit contact between the bacteria and the epithelial surfaces. In invasive disease, however, thicker capsules are required so that *S. pneumoniae* can evade phagocytosis and other immune defense mechanisms (van der Poll and Opal, 2009).

Pneumolysin (PLY), a 53 kDa pore-forming toxin, is another critical virulence factor of *S. pneumoniae*. It is produced by almost all clinical isolates. After the release of PLY from the bacterium, it binds to the cholesterol in the host membrane, where it oligomerizes in order to form large pores of up to 30 nm in diameter (Mitchell and Mitchell, 2010; van der Poll and Opal, 2009). High amounts of PLY lead to the lysis of the host cells, but it also has biological

effects at sub-lytic concentrations. These include inhibition of the cilliary action of epithelial cells, impairment of the respiratory burst of phagocytic cells, as well as activation of the complement system and cytokine/chemokine production (Hirst, et al., 2008; Maus, et al., 2004; Rubins, et al., 1996; Witzenrath, et al., 2006). Pneumococcal strains deficient for PLY tend to display reduced levels of virulence and induce less inflammation and neutrophil recruitment. Moreover, functional PLY was implicated to be required for the pneumococcal survival in the upper and lower respiratory tract as well as for the invasion into the blood stream (Hirst, et al., 2008; Kadioglu, et al., 2002; Wellmer, et al., 2002). However, serotype 1 and 8 strains carrying a non-cytolytic PLY were still isolated from cases of invasive disease. It is possible, that expression of this non-cytolytic PLY might represent an immune evasion strategy for these pneumococcal strains (Kadioglu, et al., 2008; Witzenrath, et al., 2011).

1.1.2 Epidemiology and disease

Streptococcus pneumoniae is the main cause of community-acquired pneumonia world-wide, but can also lead to meningitis, otitis media, and sepsis. Approximately 10% of adults, 20-40% of healthy children, and >60% of infants and children in daycare are colonized by pneumococci. This colonization can either be asymptomatic and disappear after a while or it can progress to invasive disease (van der Poll and Opal, 2009). An estimated 1.6 million people per year die of IPD world-wide, the majority of which are children below the age of 5 years (Lynch and Zhanel, 2010; Parsons and Dockrell, 2002; WHO, 2008). Risk factors include age <2 years and >65 years, a compromised immune system (HIV, asplenia, organ transplant recipients), poverty, restricted access to healthcare, and co-morbidities (e.g. influenza infection) (O'Brien, et al., 2009; Parsons and Dockrell, 2002). Vaccines targeting several pathogenic pneumococcal serotypes result in a serotype-specific decrease of IPD and reduce the likelihood of transmission. However, non-vaccine strains can outcompete the targeted strains. This is thus raising the possibility for new serotypes to colonize the URT (Frazao, et al., 2010).

After encountering the host, *S. pneumoniae* colonizes the nasopharynx and competes with other commensals for this niche (Lysenko, et al., 2010). Pneumococcal carriage induces the systemic and mucosal production of strain- and serotype-specific IgG antibodies, which are, however, not sufficient for bacterial clearance (Richards, et al., 2010; Simell, et al., 2009). The pneumococci are either cleared after several weeks to months or can progress to IPD. A study showed that low-invasive serotypes have a longer duration of colonization than invasive serotypes (Sleeman, et al., 2006). At the epithelium, the expression of the capsule is down-regulated and adherence factors are exposed on the surface of the pneumococcus. These factors interact with host proteins, for example with the receptor for the platelet-activating factor, which allows invasion of the pneumococci into the epithelium and into the tissue (Gould and Weiser, 2002; Thornton, et al., 2010). Moreover, the immune response of

the host against commensal bacteria in the nasopharynx contributes to the weakening of the epithelial barrier and facilitates bacterial invasion (Clarke, et al., 2011). By progressing from the nasopharynx to the lower respiratory tract or by migrating through the epithelial barrier pneumococci gain access to sterile compartments, such as lung, brain, middle ear, and blood, where they cause disease. To this date the mortality rate in adults remains at 10-30% for bacteremic pneumonia and 16-37% for meningitis. This indicates a strong need for new therapeutic strategies (Lynch and Zhanel, 2010).

1.2 The immune system

1.2.1 The innate immune system

The innate immune system is the first-line defense of a host against pathogens. It is immediately activated by germline-encoded receptors after detection of microbial invasion or tissue damage. This results in the production of pro-inflammatory cytokines, the subsequent recruitment of phago- and lymphocytes, and in the initiation of the adaptive immune response. Thus, the innate immune system is important for starting and shaping the immune response of the host. (Medzhitov and Janeway, 2000).

The innate immune response can be initiated by basically every cell type which comes in contact with microbes, microbial molecules or endogenous danger signals (see below). The specific cell type responsible depends on the type of pathogen and the type of infection. In bacterial lung infections, for example, resident macrophages might be most important for the initiation, while other cell types including dendritic cells (DCs) and epithelial cells contribute to this response. Receptors (see below) on the plasma membrane, in the endosomes, or in the cytosol of these cells then sense conserved structures of the pathogen, the “pathogen-associated molecular patterns” (PAMPs) (Chaplin, 2010; Vance, et al., 2009). After sensing PAMPs, the cells produce cytokines and chemokines in order to activate neighboring cells and to attract neutrophils and exudate macrophages. The recruited cells help to eliminate the invading pathogens through phagocytosis, secretion of antimicrobial peptides, production of reactive oxygen species/nitrogen intermediates, and formation of neutrophil extracellular traps (Papayannopoulos and Zychlinsky, 2009). The DCs migrate to the peripheral lymph nodes, where they directly stimulate T- and indirectly B-lymphocytes and initiate the adaptive immune response (Chaplin, 2010; Ryu, et al., 2010).

Another part of the innate immunity is the complement system (Skattum, et al., 2011). Soluble factors in the serum such as antibodies or lectins (e.g. mannan-binding lectin) adhere to structures on the surface of pathogens. After binding, they initiate the complement cascade and induce the lysis and/or the phagocytosis of the microbes.

A great variety of cytokines and chemokines are produced during the innate immune response that maintain, shape, and in the end terminate the defense mechanisms. The coordinated functions of the cyto-/chemokines determine the type of the following immune response.

1.2.2 Pattern recognition receptors

The innate immune response is stimulated by the activation of pattern-recognition receptors (PRRs). PRRs are present on the plasma membrane, in the endosomes, or in the cytosol. They sense conserved structures that can be either derived from microbial pathogens (pathogen-associated molecular patterns – PAMPs) (Janeway, 1989) or from damaged host cells and tissues (danger-associated molecular patterns – DAMPs) (Matzinger, 1994). PAMPs are structures that are present in a broad range of pathogens (such as cell wall components or microbial nucleic acids) and are essential for their survival and replication. This ensures that a limited number of germline-encoded receptors can detect a limited amount of conserved structures and still be effective against a broad range of microbes. DAMPs are endogenous intracellular molecules that are released upon cellular damage and activate PRRs. They generally indicate damage inside the organism without the requirement of a preceding infection; although their role in infections becomes increasingly recognized (Horvath, et al., 2011).

1.2.2.1 Toll-like receptors

Toll-like receptors (TLRs) are conserved in vertebrates, insects, and nematodes and are composed of 10 members in humans and 12 members in mice. They can either be localized on the plasma membrane (TLR1, -2, -4, -5, -6) or in the endosomal compartment (TLR3, -7, -8, -9). TLR4 can relocate from the plasma membrane to the endosomes after stimulation (O'Neill and Bowie, 2007). TLRs are composed of an extracellular ligand-binding domain and an intracellular signaling domain. Upon ligand binding, the TLRs homo- or heterodimerize and activate downstream signaling pathways. Most TLRs are known to homodimerize, but e.g. TLR2 can form heterodimers with either TLR1 or TLR6. Bacterial cell wall components, such as peptidoglycans are sensed by TLR2 homodimers (Schroder, et al., 2003).The TLR2 heterodimers TLR1/2 and TLR2/6 are able to recognize tri- and diacetylated lipopeptides, respectively (Takeda, et al., 2002; Takeuchi, et al., 2002). TLR4 senses lipopolysaccharide (LPS) of gram-negative bacteria (Poltorak, et al., 1998) and is also discussed to recognize pneumococcal PLY (Malley, et al., 2003). TLR5 is activated by flagellin (Hayashi, et al., 2001). The endosomal TLRs are activated by nucleic acids, such as single-stranded RNA (TLR-7, -8) (Heil, et al., 2004), double-stranded RNA (TLR3) (Alexopoulou, et al., 2001), or unmethylated CpG motifs in double-stranded DNA (TLR9) (Hemmi, et al., 2000).

The stimulation of TLRs induces the recruitment of adaptor molecules (MyD88, Mal, TRIF, TRAM), which subsequently initiate pro-inflammatory signaling cascades. MyD88 is a central adaptor molecule for all TLRs (except TLR3) and is also recruited to the IL-1 receptor. It mediates the formation of a signaling complex that activates the transcription factor NF-κB as well as MAP kinases and leads to the production of cytokines and chemokines (O'Neill and Bowie, 2007). Children deficient in MyD88 or a component of its signaling complex (IRAK4) suffer from recurrent cases of IPD (Picard, et al., 2010). Mal is a supporting adaptor for MyD88-dependent signaling that acts as a connecter between TLR2 or -4 and MyD88. TRIF is the adaptor molecule for TLR3 and TLR4 and activates NF-κB as well as the IRF3 transcription factor, which leads to the production of type I interferons (IFNs). Type I IFNs are important in antiviral and antibacterial defenses (see below). Mice deficient in TRIF had an impaired immune response to lung infections of the gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia Coli* characterized by diminished cytokine/chemokine production and neutrophil recruitment (Cai, et al., 2009; Jeyaseelan, et al., 2007). Moreover, TRIF-/- mice infected with *Klebsiella pneumoniae* had a decreased survival and bacterial clearance (Cai, et al., 2009). TRAM is the fourth adaptor molecule of the TLRs and connects TLR4 to TRIF thus allowing TLR4 to activate IRF transcription factors (O'Neill and Bowie, 2007). Thus, activation of TLR4 on the cell surface triggers an early response of NF-κB activation via MyD88 and MAL, whereas in the late phase TLR4 shuttles to the endosome and activates NF-κB and IRF transcription factors via the adaptors TRAM and TRIF (Palsson-McDermott and O'Neill, 2004).

1.2.2.2 NOD-like receptors

NOD-like receptors (NLRs) are cytosolic PRRs that can be functionally divided into two groups: one group is activating pro-inflammatory signaling pathways leading to the stimulation of NF-κB and MAP kinases, whereas the other group is forming inflammasomes (Chen, et al., 2009). Inflammasomes are multi-protein complexes that activate caspase-1, which subsequently cleaves the cytokines pro-IL-1β and pro-IL-18 into their mature and active forms (Schroder and Tschopp, 2010).

NOD1 and NOD2 belong to the group of NLRs that activate NF-κB and MAP kinase pathways. NOD1 senses muropeptides of gram-negative and some gram-positive bacteria, whereas NOD2 detects muramyl dipeptides of both gram-negative and gram-positive bacteria (Chen, et al., 2009). Stimulation of these NLRs leads to the phosphorylation of the RIP2 kinase and to the subsequent activation of NF-κB and MAP kinases. This results in the production of pro-inflammatory cytokines. Additionally to activating the above mentioned pathways, NOD2 was also implicated in the up-regulation of type I IFNs: a recent study

demonstrated that it interacts with single-stranded viral RNA leading to the activation of the adaptor molecule MAVS and to the subsequent induction of IFNβ (Sabbah, et al., 2009).

NOD1 and NOD2 not only sense PAMPs of cytosolic, but also of extracellular pathogens, because extracellular ligands can be delivered into the cytosol via transporter systems. A recently described transporter system is the oligopeptide transporter hPepT1, which is able to transfer bacterial peptides, such as fMLP and MDP, into the cytosol (Charrier and Merlin, 2006). After reaching the cytosol via hPepT1, MDP was shown to stimulate the activation of NF-κB via NOD2. This oligopeptide transporter is expressed in the intestinal epithelium, but also on human monocytes and could therefore have a central function in the activation of NOD2 by extracellular pathogens (Charrier, et al., 2006). Moreover, other pathways for PAMPs to gain access to the cytosol have been described. Pore-forming toxins from some bacterial strains could damage the plasma or endosomal membranes by forming large pores. These in turn could subsequently allow extracellular PAMPs to gain access to the cytosol, as has been suggested for group B streptococcus (Charrel-Dennis, et al., 2008) and for *S. pneumoniae* during nasopharyngeal colonization (Davis, et al., 2011). Furthermore, type IV secretion systems have been described to transport bacterial components that could potentially act as PAMPs (Lippmann, et al., 2011; Nagai and Roy, 2003). Thus, several transport mechanisms have been described that translocate PAMPs from the extracellular space into the cytosol. It is conceivable that further mechanisms exist to provide the manifold intracellular PRRs with pathogen-derived ligands.

The other group of NLRs is known to form inflammasomes. Inflammasomes are composed of an NLR (for example NLRP3), pro-caspase-1, and the adaptor molecule ASC, although the latter can be dispensable for some inflammasomes (Schroder and Tschopp, 2010). Upon stimulation of the NLR, the inflammasome protein complex assembles and pro-caspase-1 is autoproteolytically cleaved into its active form. The active caspase-1 can subsequently cleave the inactive pro-forms of some cytokines, e.g. pro-IL-1β and pro-IL-18 into their biologically active forms (de Veerdonk, et al., 2011). The NLRP3 inflammasome can be activated by various stimuli not only after infection with pathogens, but also in response to endogenous danger signals (DAMPs) or xenocompounds such as asbestos or aluminum hydroxide. This suggests that NLRP3 itself is not a PRR but indicates the existence of several sensing pathways that culminate in the activation of the NLRP3 inflammasome (Leemans, et al., 2011).

Moreover, inflammasomes mediate a caspase-1-dependent programmed cell death known as pyroptosis, which is involved in restricting the replication of some intracellular pathogens (Miao, et al., 2011). Thus, inflammasomes are important regulators of the pro-inflammatory response and have a central role in the host defense.

1.2.2.3 RIG-I-like receptors

The group of RIG-I-like receptors (RLRs) is composed of three members whose function is the recognition of cytosolic RNA via their DExD/H box RNA helicase domain (Loo and Gale, 2011). RIG-I (DDX58) senses short ssRNA and dsRNA with a 5' phosphate end, whereas MDA5 detects longer dsRNA substrates. After sensing RNA, RIG-I and MDA5 recruit the adaptor molecule MAVS. MAVS then activates the transcription factors IRF3 and NF-κB and consequently leads to the induction of type I IFNs and pro-inflammatory cytokines (Loo and Gale, 2011). Another signaling cofactor of RIG-I can be the adaptor molecule STING, which is also involved in the signaling pathways activated by cytosolic DNA (Ishikawa and Barber, 2008).

Due to their ability to detect various types of cytosolic RNA, RLRs play an important role in the cellular defense against many viruses. The activation of the type I IFN response induces an anti-viral state in the infected and non-infected neighboring cells. Knockout mice deficient in RIG-I or MDA5 are more susceptible to infection with various RNA viruses (Kato, et al., 2006). In addition, RLRs have a role in the detection of cytosolic DNA. It was demonstrated that cytosolic AT-rich DNA is converted to RNA by RNA polymerase III (Ablasser, et al., 2009; Chiu, et al., 2009). This RNA is a ligand for RIG-I and induces the production of type I IFNs. The RNA polymerase III/RIG-I system thus functionally belongs to the cytosolic DNA sensing pathways (see also below).

1.2.2.4 Cytosolic DNA sensors

1.2.2.4.1 DAI

The detection of cytosolic DNA is mediated by different systems. DAI (DNA-dependent activator of IFN regulatory factors; also known as ZBP-1) was the first cytosolic DNA sensor described that leads to the induction of IFNα/β (Takaoka, et al., 2007). However, macrophages or mouse embryonic fibroblasts from DAI-deficient mice did not show a defect in the induction of type I IFNs after DNA stimulation (Ishii, et al., 2008). Moreover, the siRNA-mediated knockdown of DAI in an alveolar epithelial cell line did not influence the type I IFN response after infection with *L. pneumophila (*Lippmann, et al., 2008). This lack of a phenotype could be explained by the existence of several redundant DNA-sensing pathways in the cytosol, which lead to the transcription of type I IFNs.

1.2.2.4.2 PYHIN proteins

The PYHIN protein AIM2 belongs to the HIN200-domain family of proteins and has been recently described to sense cytosolic DNA (Burckstummer, et al., 2009; Fernandes-Alnemri, et al., 2010; Hornung, et al., 2009; Roberts, et al., 2009). AIM2 contains a HIN200-domain that mediates the DNA recognition and a pyrin domain that interacts with the inflammasome adaptor molecule ASC. Activation of AIM2 leads to the formation of an inflammasome and to

the subsequent production of mature IL-1β. AIM2 was shown to be involved in the host defense against DNA viruses (Rathinam, et al., 2010), the gram-negative bacterium *Francisella tularensis* (Fernandes-Alnemri, et al., 2010; Jones, et al., 2010), as well as the gram-positive bacterium *L. monocytogenes* (Kim, et al., 2010; Sauer, et al., 2010; Tsuchiya, et al., 2010; Warren, et al., 2010). Thus, AIM2 is playing an important role in the host defense against a broad range of pathogens.

IFI16 is another member of the PYHIN protein family and is also able to sense DNA via its HIN200 domain. It was first described to mediate the induction of type I IFNs after transfection of DNA and after infection with DNA viruses, e.g. herpes virus (Unterholzner, et al., 2010). Moreover, it has been indicated that IFI16 might also have direct antiviral effects (Gariano, et al., 2012). Recent publications indicate additional functions of IFI16 in inhibiting the AIM2 inflammasome (Veeranki, et al., 2011) and in forming an inflammasome itself in and around the nucleus in response to Kaposi Sarcoma-associated herpesvirus infection (Kerur, et al., 2011). Thus, IFI16 is playing a multifaceted role in the host defense by stimulating both a type I IFN response as well as the production of inflammasome-dependent cytokines. For the induction of type I IFNs, IFI16 recruits the adaptor protein STING, which in turn relays the signal to activate the transcription factors IRF-3 and NF-κB. STING plays a central role in the defense against many different pathogens. Mice deficient in STING were more susceptible to infection with herpes simplex virus (Ishikawa, et al., 2009). Moreover, STING was essential for the type I IFN response after infection with *L. monocytogenes* (Ishikawa, et al., 2009; Sauer, et al., 2011) and *L. pneumophila* (Lippmann, et al., 2011). Apart from being involved in pathways that sense cytosolic DNA, STING has been recently shown to act as a PRR that detects and directly binds to cyclic di-GMP, which also results in the induction of type I IFNs (Burdette, et al., 2011). Cyclic di-GMP is a prevalent second messenger molecule in many bacteria that controls processes, such as flagellar motility, biofilm formation, and cell cycle regulation (Mills, et al., 2011). Thus, STING is a central protein in the initiation of the type I IFN response after detection of different PAMPs in the cytosol.

1.2.2.4.3 RIG-I

Even though the helicase RIG-I (DDX58) can only directly sense RNA molecules in the cytosol, it has also been implicated in the recognition of DNA (Ablasser, et al., 2009; Chiu, et al., 2009). Poly dA:dT DNA was shown to be converted into an RNA intermediate by the RNA polymerase III in the cytosol. This intermediate can be then sensed by RIG-I, which leads to the induction of type I IFNs via the pathway described above (see 1.2.2.3).

In conclusion, cytosolic DNA is sensed by different receptor systems that mostly converge in the activation of the transcription factors IRF-3 and/or IRF-7 as well as NF-κB and

consequently lead to the activation of type I IFNs and pro-inflammatory cytokines. However, some are able to form inflammasomes after sensing DNA and contribute to the production of IL-1β and IL-18.

1.2.3 Innate immune recognition of *S. pneumoniae*

1.2.3.1 Toll-like receptors

The innate immune response to *S. pneumoniae* has been investigated during the last years. The membrane-bound TLRs as well as cytosolic PRRs were shown to be involved in the host defense against pneumococci (see below). After infection with *S. pneumoniae* TLR2, -4, and -9 have been shown to play a role in the recognition of this pathogen (Albiger, et al., 2007; Knapp, et al., 2004; Malley, et al., 2003). An overview which TLR pathways contribute to sensing *S. pneumoniae* and what ligands they recognize is given in Fig. 1.

TLR2 is the most thoroughly investigated TLR in pneumococcal infection. This receptor senses components of the cell wall of *S. pneumoniae*, such as peptidoglycan and lipoteichoic acid, in combination with the co-receptors CD14 and the lipopolysaccharide-binding protein (Schroder, et al., 2003; Schwandner, et al., 1999). Stimulation of TLR2 by *S. pneumoniae in vitro* led to the activation of NF-κB and to the production of cytokines and chemokines, such as TNFα, IL-6, IL-8, and KC (Knapp, et al., 2004; Mogensen, et al., 2006). *In vivo*, the role of TLR2 has been investigated in different models of pneumococcal infection. In a model of pneumococcal colonization, TLR2-/- mice had an increased bacterial burden of pneumococci during later stages of infection compared to wild-type mice (van Rossum, et al., 2005). Moreover, TLR2 has a pronounced role in *S. pneumoniae*-induced meningitis. TLR2-/- mice were more susceptible to disease than wild-type mice and had an increased disease progression and pneumococcal replication. Additionally, they succumbed earlier to the infection. Interestingly, the early influx of leukocytes into the cerebrospinal fluid also seemed to be dependent on TLR2 (Echchannaoui, et al., 2002; Koedel, et al., 2003). In a model of *S. pneumoniae* pneumonia, TLR2-/- mice displayed slightly reduced inflammation, cytokine levels and neutrophil influx (Knapp, et al., 2004). Surprisingly, a difference in mortality between the wild-type and knockout mice could only be observed after infection with PLY-deficient pneumococci and not with the wild-type strain (Dessing, et al., 2008).

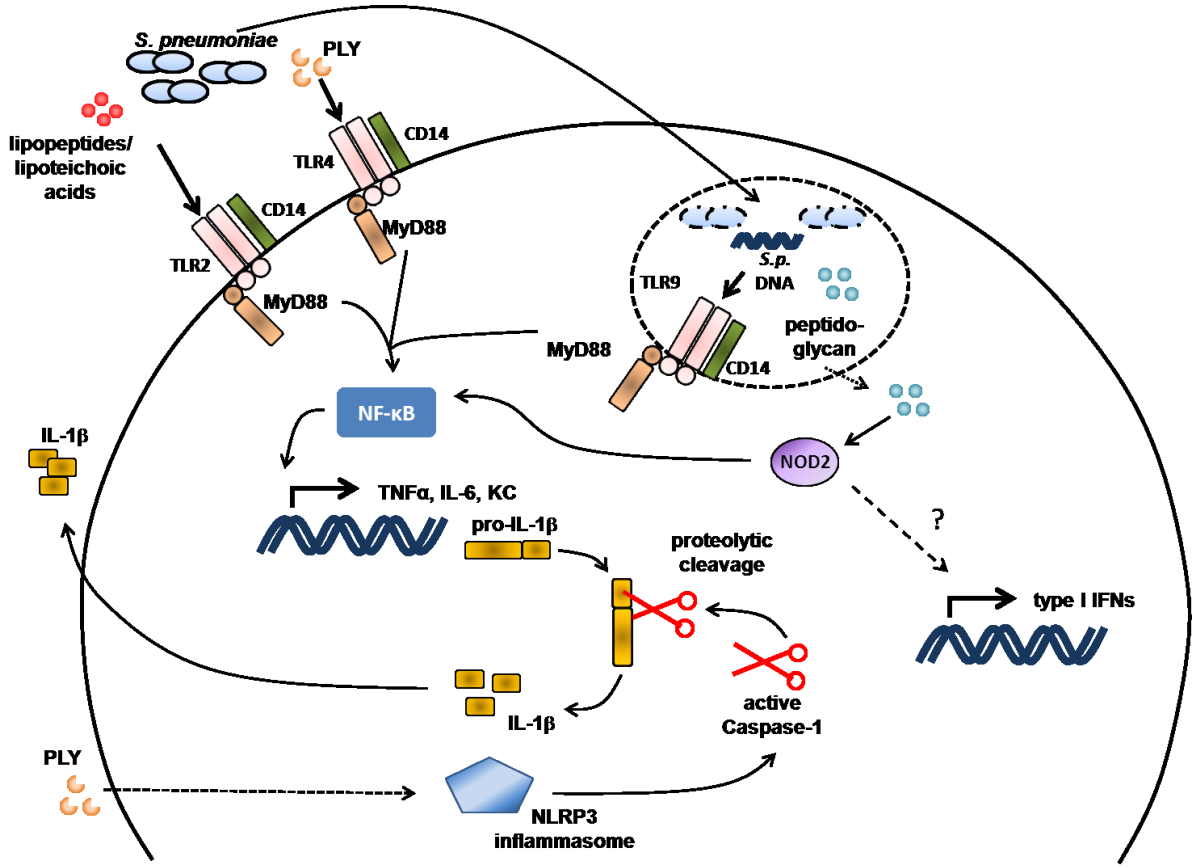

Fig. 1: Recognition of *S. pneumoniae* by the innate immune system. Pneumococcal components, such as lipopeptides, PLY, or DNA are sensed by Toll-like receptors (TLRs) on the plasma membrane or in the endosomal compartment. Moreover, some pneumococcal cell wall components such as peptidoglycan can access the cytosol, where they are sensed by NOD2. All these receptors induce the activation of the transcription factor NF-κB and consequently lead to the production of cytokines and chemokines, such as TNFα, IL-6, KC, and pro-IL-1β. The latter is further processed into its mature and active form by inflammasomes that contain active caspase-1. The NLRP3 inflammasome is activated in response to pneumococcal PLY. In addition to stimulating NF-κB, NOD2 was also recently reported to contribute to type I IFN induction during pneumococcal colonization. To this date, it remains to be elucidated if other pneumococcal components are able to access the cytosol and activate innate immune pathways.

The exact role of TLR4, the well-described receptor for LPS of gram-negative bacteria, during infection with *S. pneumoniae* remains controversial. *In vivo* studies demonstrated that TLR4-/- mice had a reduced survival and an enhanced bacterial replication during early time points of colonization and after pulmonary infection with small doses of *S. pneumoniae* (Branger, et al., 2004; Malley, et al., 2003). This could, however, not be observed in a model of pneumococcal sepsis (Benton, et al., 1997). Some studies implicated that pneumococcal PLY is a cognate ligand of TLR4, which is able to induce cytokine production (Malley, et al., 2003; Srivastava, et al., 2005). However, others challenge this model by demonstrating a TLR4-independent cytokine production by PLY (McNeela, et al., 2010) and by demonstrating that TLR4 might also recognize DAMPs after acute lung injury (Imai, et al., 2008). The *in vivo* data clearly demonstrate a role of TLR4 during pneumococcal infection. However, the exact mechanism of TLR4 activation by *S. pneumoniae* requires further investigation.

TLR9 is the third toll-like receptor known to be involved in pneumococcal recognition. It is activated after sensing unmethylated CpG sequences in *S. pneumoniae* DNA. Hek-293 cells overexpressing TLR9 were responsive to both live pneumococci and pneumococcal DNA, resulting in the activation of NF-κB and the production of IL-8 (Mogensen, et al., 2006). Mice deficient in TLR9 had an impaired survival after intranasal, but not after intra-peritoneal challenge with *S. pneumoniae*. The bacteria were found to have an increased replication in the bronchi of TLR9-/- mice and also an increased dissemination into the lung tissue. The cytokine production in this model, however, was similar to the one in wild-type mice (Albiger, et al., 2007).

As described above, the single TLR knockout mice did not show a severe phenotype after pneumococcal infection indicating a redundancy in TLR signaling on the activation of the innate immune response (Albiger, et al., 2007; Knapp, et al., 2004; Malley, et al., 2003). However, deficiency in MyD88, the central adaptor of TLR signaling and IL-1 receptor signaling, severely impaired the immune response to *S. pneumoniae* and greatly enhanced the susceptibility of the knockout mice to invasive disease (Albiger, et al., 2005; Khan, et al., 2005; Koedel, et al., 2004). This indicates the existence of multiple yet partly redundant MyD88-depedent pathways that collectively activate the immune response.

1.2.3.2 NOD-like receptors

Bacterial components of *S. pneumoniae* are also sensed in the cytosol. After pneumococcal infection, NOD2 is stimulated, which triggers the activation of NF-κB (Opitz, et al., 2004) (see Figure 1). NF-κB subsequently induces the production of pro-inflammatory cytokines and chemokines. *In vivo*, NOD2 was involved in the initiation of the innate immune response via mediating the MCP-1-dependent recruitment of macrophages during pneumococcal colonization (Davis, et al., 2011). Moreover, this NLR also supports the activation of the adaptive immune response by contributing to the induction of antibody production. Additionally to a function during colonization of the nasopharynx, NOD2 was also shown to be important in a model of *S. pneumoniae* meningitis (Liu, et al., 2010). In contrast to their wild-type counterparts, mice deficient in NOD2 were found to have reduced levels of astrogliosis, demyelination, and inflammation. Accordingly, microglia and astroglia from NOD2-/- mice were found to secrete diminished amounts of TNFα, IL-6 and MIP-1α after *in vitro* infection with *S. pneumoniae*.

Additionally to NOD2, another NLR was demonstrated to play a role in the innate immune response to *S. pneumoniae*. NLRP3 was activated by pneumococci, formed an inflammasome, and was thus contributing to the production of IL-1β by macrophages and DCs (Fang, et al., 2011; Hoegen, et al., 2011; McNeela, et al., 2010; Witzenrath, et al., 2011). This activation was shown to be dependent on pneumococcal PLY. *In vivo*, NLRP3-/-

mice had a reduced pneumococcal clearance in the lung (McNeela, et al., 2010) as well as an impaired lung endothelial/epithelial barrier (Witzenrath, et al., 2011). In contrast, NLRP3 knockout mice showed a reduced pathology and improved clinical scores in a model of pneumococcal meningitis (Hoegen, et al., 2011). This indicates a differential role of the NLRP3 inflammasome in different compartments of the organism during infection. Interestingly, significant levels of IL-1β were still detectable in NLRP3-/- cells and mice after pneumococcal infection (Fang, et al., 2011; McNeela, et al., 2010; Witzenrath, et al., 2011). Thus, other inflammasomes are likely to contribute to the IL-1β response during infection with *S. pneumoniae*.

1.2.4 Innate immune effectors

1.2.4.1 Type I interferons

Type I interferons (IFNs) are a group of 14 IFNα genes, 1 IFNβ gene, and several other still poorly characterized members (e.g. IFNε or IFNκ) (Decker, et al., 2005). They are produced during infections with viral or bacterial pathogens (Monroe, et al., 2010). The various cellular pathways leading to the induction of type I IFNs are summarized in Figure 2. Stimulation of the membrane-bound TLRs or various cytosolic nucleic acid sensors induces the recruitment of adaptor molecules (MyD88, STING, TRIF, MAVS) and the subsequent activation of the transcription factors IRF-3 and/or IRF-7. These bind to the promoter regions of type I IFN genes and induce the transcription, production, and secretion of these cytokines. Moreover, the promoter region of the IFNβ gene contains additional binding sites for the transcription factors NF-κB (Thanos and Maniatis, 1992) and ATF2/c-jun (Du, et al., 1993). The coordinated binding of all transcription factors in the promoter region cooperatively stimulates a strong transcription of the IFNβ gene (Wathelet, et al., 1998).

After secretion, type I IFNs bind to the IFNα/β receptor (IFNAR) and activate downstream signaling cascades that involve the Jak-Stat pathway (de Weerd, et al., 2007). This leads to the formation of the ISGF3 complex consisting of STAT1, STAT2 and IRF9, which acts as a transcription factor and binds to ISRE sites in the promoters of interferon-stimulated genes (ISGs). Moreover, IFNAR activation also induces the activation of GAF, a Stat-1 homodimer, that binds to the GAS elements in ISG promoter regions (Decker, et al., 2005).

Type I IFNs have a broad range of biological effects that modulate the immune response to various pathogens. In some infections this can be beneficial, whereas in others the overall effect of IFNα/β is detrimental to the host. The first described effect of type I IFNs was its ability to confer an “anti-viral state” to infected and non-infected neighboring cells (Isaacs and Lindenmann, 1957). This is achieved by inducing anti-viral ISGs, such as Mx proteins, IFITM proteins, and Viperin, which inhibit the intracellular viral replication (Liu, et al., 2011).

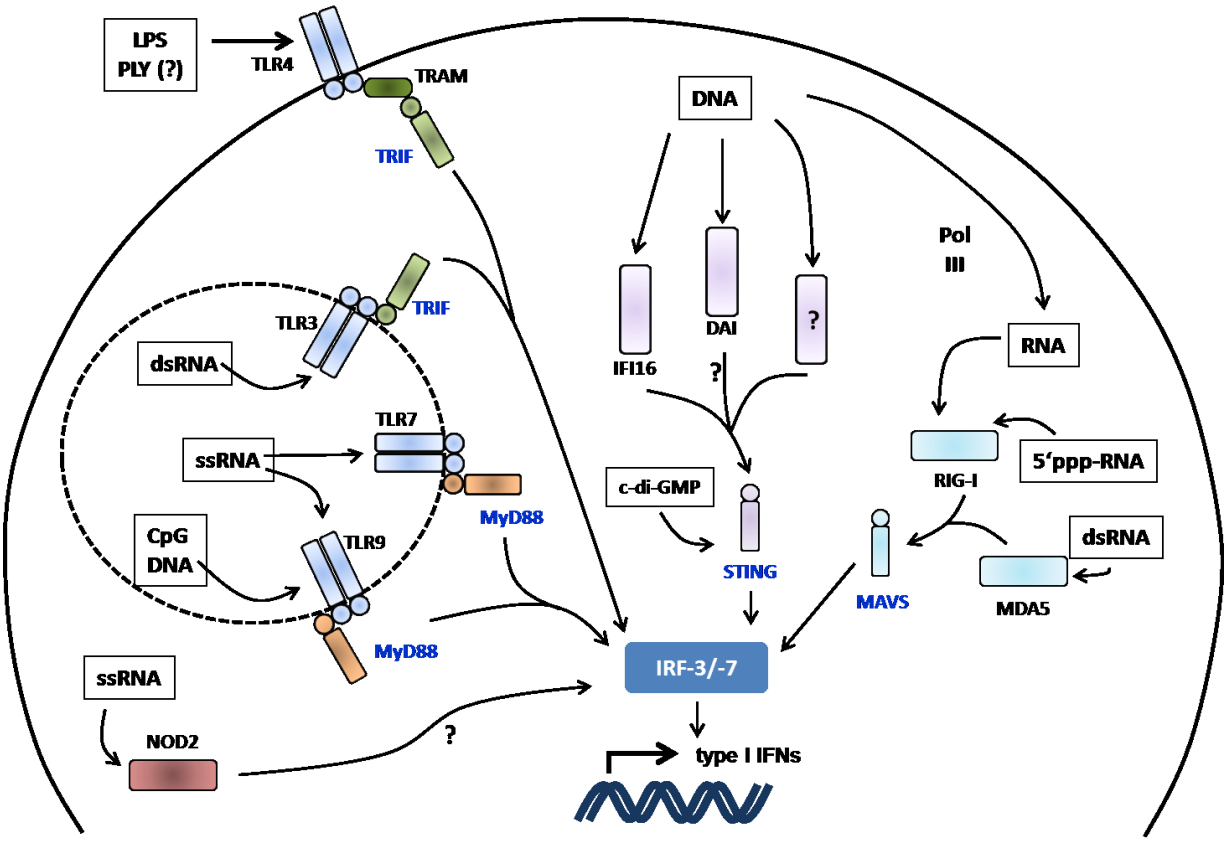

Fig. 2: Cellular signaling pathways that lead to the induction of type I IFNs. TLRs on the plasma membrane or in the endosome recognize microbial components or nucleic acids and signal via the adaptor proteins MyD88 or TRIF to activate IRF-3/-7 transcription factors. NOD2 and RLRs can sense RNA components and activate type I IFNs via IRF-3/-7. RLRs signal via the adaptor proteins MAVS. DNA is sensed by a diverse group of cytosolic receptors. Most signal via the adaptor protein STING, although also STING-independent pathways (e.g. DNA polymerase III/RIG-I pathway) have been described. Moreover, STING is also able to directly sense cyclic di-GMP (c-di-GMP). All DNA sensors finally activate the transcription factors IRF-3 and/or IRF-7, which in turn stimulate the transcription of type I IFNs

Moreover, type I IFNs also induce ISGs with anti-bacterial functions. A prominent example for this is the inducible nitric oxide synthase (iNOS), which has been shown to be positively regulated by IFNα/β, which has protective effects against pathogens such as *Chlamydia trachomatis* (Devitt, et al., 1996) and *Burkholderia pseudomallei* (Utaisincharoen, et al., 2003). Moreover, type I IFNs (often together with type II IFN) induce the enzyme indoleamine 2,3-dioxygenase that restricts the availability of tryptophan (Puccetti, 2007) and thus inhibits the growth of pathogens including *Streptococci*, *Enterococci*, and *Chlamydophila pneumoniae* (Daubener and MacKenzie, 1999; Njau, et al., 2009). Additionally, a group of p47 GTPases, also known as the IRG proteins, were shown to be important for the defense against intracellular pathogens, e.g. *L. pneumophila* and *Toxoplasma gondii* (Howard, 2008). Early studies reported that type I IFNs are able to inhibit the invasion of *Salmonella* and *Shigella* bacteria into epithelial cells (Bukholm, et al., 1984; Niesel, et al., 1986). This indicates yet another protective effect of IFNα/β against some bacteria. However, the exact mechanism of this inhibition remains to be elucidated.

Additionally to inducing a number of anti-viral and anti-bacterial ISGs, type I IFNs sensitize cells to undergo apoptosis. This effect has been described for macrophages and lymphocytes during infection with *Listeria monocytogenes* (Carrero, et al., 2004; Stockinger, et al., 2002). While in viral infection, type I IFN-mediated apoptosis of infected host cells could limit the proliferation of the virus, during infection with *Listeria*, this mechanism promotes the death of important immune cells and is detrimental to the host (Carrero and Unanue, 2012).

Type I IFNs also influence the expression of several cytokines. They were shown to stimulate the production of type II IFN, also known as IFNγ, together with IL-18 during infection with *Salmonella typhimurium* (Freudenberg, et al., 2002). Moreover, a contribution of type I IFNs to IFNγ production was also observed in mice infected with *L. monocytogenes* (Carrero, et al., 2006). Additionally to influencing the secretion of type II IFN, IFNα/β also down-regulates the surface expression of the IFNγ receptor (IFNGR) on immune cells, including macrophages and dendritic cells (Rayamajhi, et al., 2010). This renders the cells less responsive to activation by IFNγ and consequently modulates the immune response. Additionally to type II IFN, the cytokine IL-1β is also regulated by type I IFNs on different levels. Stimulation of cells with IFNα/β led to reduced mRNA-levels of the precursor pro-IL-1β (Guarda, et al., 2011; Novikov, et al., 2011), which was proposed to occur via the induction of the anti-inflammatory cytokine IL-10. Moreover, type I IFNs appeared to diminish the post-translational processing of pro-IL-1β by inhibiting the NLRP1 and NLRP3 inflammasomes (Guarda, et al., 2011). However, IFNα/β does not only have inhibiting effects on inflammasomes since it was shown to induce the AIM2 inflammasome, which was required for defense against *Francisella* infection (Choubey, et al., 2010; Fernandes-Alnemri, et al., 2010). Thus, depending on the type of infection type I IFNs can have both inhibiting and stimulating effects on IL-1β.

Apart from regulating the production of cytokines, the influence of IFNα/β on the expression of several chemokines has also been demonstrated. It was shown that the chemokine MCP-1 was induced and required for the recruitment of macrophages during infection with *L. monocytogenes* and *S. pneumoniae* (Antonelli, et al., 2010; Davis, et al., 2011; Jia, et al., 2009). However, in the model of *S. pneumoniae* infection, the expression of this chemokine was inhibited by high levels of type I IFNs elicited by a preceding influenza infection (Nakamura, et al., 2011). Moreover, an inhibiting effect of IFNα/β could be observed for the neutrophil-recruiting chemokines KC and MIP-2 in a similar model (Shahangian, et al., 2009).

Type I IFNs also influence the T cell response during infection. It was described that they negatively regulate the production of the cytokine IL-17 produced by the subset of γδ-T cells (Henry, et al., 2010). This was linked to a reduced recruitment of neutrophils in an infection

with *Francisella*. In accordance, type I IFNs were shown to induce IL-27, a negative regulator of IL-17 production (Guo, et al., 2008).

In vivo, Type I IFNs are required in the defense against many viruses (Bogdan, 2000; Zhang, et al., 2008). Moreover, their role in the defense against some bacteria has been begun to be characterized during the last years. Type I IFNs had protective effects for the host in a murine model of *L. pneumophila* pneumonia (Lippmann, et al., 2011). Moreover, they were required in the defense against systemic infection with group B streptococcus and *E. coli* (Mancuso, et al., 2007). However, in other bacterial infections, such as *L. monocytogenes* or *Mycobacterium tuberculosis*, the induction of type I IFNs contributed to a worse outcome for the host (O'Connell, et al., 2004; Stanley, et al., 2007). IFNAR-/- mice infected with *Listeria* had reduced bacterial loads in the liver and the spleen as well as a strongly enhanced survival compared to wild-type mice (O'Connell, et al., 2004). Similarly, after infection with *M. tuberculosis*, these knockout mice had fewer bacteria in the spleen than their wild-type counterparts (Stanley, et al., 2007). Thus the overall effect of type I IFNs on the host defense *in vivo* varies in different infection models.

1.2.4.2 Type II interferon

Type II interferon, also known as IFNγ, mediates different effects in the innate and the adaptive immune response. Producers of IFNγ include natural killer T cells and other T lymphocytes, neutrophils, macrophages, and dendritic cells (Schroder, et al., 2004; Yamada, et al., 2011). Whereas the local production of IFNγ by natural killer T cells, macrophages, and dendritic cells may be important in the early activation of infected and non-infected neighboring cells, T lymphocytes are the main producers of type II IFN during the following adaptive immune response (Brigl, et al., 2011; Schroder, et al., 2004). The induction of type II IFN occurs via the interleukin IL-12, which is secreted by e.g. DCs after infection. IL-12 induces IFNγ production in the recruited neutrophils, natural killer T cells, and other T lymphocytes. IL-18 is secreted by APCs after inflammasome activation and further enhances the production of IFNγ (Schroder, et al., 2004).

After secretion, IFNγ binds to the IFNγ receptor (IFNGR), which is composed of two homodimers (IFNGR1 dimer and IFNGR2 dimer). This binding activates a downstream signaling cascade leading to the JAK-dependent phosphorylation of transcription factors, e.g. Stat-1. However, the activation of JAK-Stat independent pathways after stimulation with IFNγ has also been described (Gough, et al., 2008). Stat-1 homodimerizes to form the GAF transcription factor, then translocates into the nucleus, and finally stimulates the transcription of a variety of interferon-stimulated genes that contain GAS elements in their promoter regions. Moreover, type II IFN can also induce the ISGF3 complex (see above), which leads to the transcription of genes with ISRE elements in their promoters (Morrow, et al., 2011).

Thus, there is a substantial overlap in ISGs induced by type I and type II IFNs. However, both types of IFN also stimulate the expression of some other ISGs exclusively (Schroder, et al., 2004).

Type II IFN has a broad variety of functions in activating the innate immune system and shaping the adaptive immune response. It induces chemokines and adhesion molecules that attract immune cells to the site of infection (Gil, et al., 2001; Rollins, et al., 1990; Schroder, et al., 2004). Recruited neutrophils and macrophages are activated by IFNγ to produce reactive oxygen species (ROS) and reactive nitrogen intermediates, which mediate the killing of both intracellular and extracellular pathogens. Moreover, type II IFN (as well as type I IFNs) can prime the recruited neutrophils to promote the formation of Neutrophil Extracellular Traps (NETs) that have a broad anti-microbial function (Martinelli, et al., 2004; Papayannopoulos and Zychlinsky, 2009). Additionally, IFNγ leads to the enhancement of phagocytosis neutrophils and antigen presentation on MHCI and MHCII complexes. IFNγ also leads to the induction of antiviral ISGs, such as PKR. In terms of shaping the adaptive immune response, type II IFN promotes the development of a Th1 response (Schroder, et al., 2004).

Mice deficient in IFNγ signaling show an enhanced susceptibility to viral, bacterial, and parasitic infections (Shtrichman and Samuel, 2001). Type II IFNs contribute to anti-viral immunity, e.g. against hepatitis B virus (Guidotti, et al., 2000) and herpes simplex virus (Cantin, et al., 1999). However, their role in the anti-microbial defense against intracellular pathogens is much more prominent. Mice deficient in IFNγ signaling were more susceptible to infection with various pathogens including *L. monocytogenes* (Huang, et al., 1993), *S. typhimurium* (Mastroeni, et al., 1999), and *M. tuberculosis* (Flynn, et al., 1993). Moreover, type II IFN was shown to be important for the immune response to parasitic infections with *Leishmania major* (Wang, et al., 1994). Thus, IFNγ plays an important part in mediating the immune response against a wide range of pathogens.

1.3 Immune response to bacterial pneumonia

The immune system has developed a vast array of mechanisms to sense and eliminate invading pathogens. Many pathogens that cause bacterial pneumonia, such as *S. pneumoniae* and *Haemophilus influenza,* can colonize the nasopharynx. One way of the host to counteract this colonization is the production of mucus. The mucus acts as a mechanical barrier, but also contains anti-microbial peptides and proteins (defensins, lysozyme, lactoferrin) as well as ROS (Ryu, et al., 2010). Moreover, the cilia on the epithelium induce a constant flow of the mucus towards the mouth and out of the airways. Additionally, the colonization of the nasopharynx induces immediate innate immune responses via PRRs including TLRs and NLRs, which leads to the expression of chemokines, such as KC, MCP-1, or RANTES (Bootsma, et al., 2007; Davis, et al., 2011; Palaniappan, et al., 2006). These

chemokines recruit and/or activate cells of the innate (neutrophils, monocytes/macrophages) and adaptive (T cells, B cells) immune system that mediate the clearance of the bacteria.

Defects in the recruitment process of immune cells, but also the impairment of the epithelial barrier integrity during extravasation of phagocytes present opportunities for the bacteria in the nasopharynx to invade into the surrounding tissue and to access the sterile lower respiratory tract (LRT) (Clarke, et al., 2011; Palaniappan, et al., 2006). In the LRT, bacteria encounter several antimicrobial mechanisms that assist in clearance of the pathogens. Type II alveolar epithelial cells produce surfactant proteins that have anti-bacterial functions. Surfactant protein D can interact with and aggregate microbes, which makes them more accessible to neutrophil phagocytosis or mucocilliary clearance (Hartshorn, et al., 1998). Moreover, microbe-bound surfactant proteins A and D are able to activate alveolar macrophages (Gardai, et al., 2003). Additionally to the binding of surfactant proteins, components of the complement system opsonize invading pathogens. These can involve the binding of the complement protein C1q to secretory IgM antibodies bound to bacteria (classical pathway) or of the mannan binding lectin to carbohydrate moieties on the surface of microbes (lectin pathway). Activation of the complement pathway enhances the phagocytosis of the opsonized bacteria and/or leads to their direct lysis. Some complement factors have chemotactic properties and recruit phagocytic cells (Skattum, et al., 2011).

Apart from being opsonized by surfactant proteins and complement factors, bacteria are directly sensed by alveolar macrophages and dendritic cells via PRRs. This leads to the activation of NF-κB and IRF transcription factors in these cells and to the production of pro-inflammatory cytokines including IL-1β, TNFα, and IL-12 (Mizgerd, 2008). Invariant Natural killer T cells (iNKT cells) contribute to the innate immune activation by sensing bacterial glycolipids presented on APCs (Boyton, 2008; Kinjo, et al., 2011). This leads to the production of IFNγ and IL-17A. After secretion, the cytokines IL-1β, TNFα, and IL-17A activate the NF-κB pathway in neighboring alveolar epithelial cells. This NF-κB activation (in both immune and epithelial cells) leads to the production of chemokines (KC, MIP-2, CXCL5) attracting neutrophils to the site of infection. Moreover, TNFα regulates e.g. the expression of neutrophil-adhesion molecules on the endothelium (Craig, et al., 2009).

Stimulation of PRRs induces also the production of other cytokines, such as type I IFNs. These induce many anti-viral and anti-bacterial ISGs and mediate immunity against some intracellular bacteria (Lippmann, et al., 2011). Moreover, GM-CSF is also produced in a TLR4-dependent manner (Standiford, et al., 2011). GM-CSF is a growth factor that stimulates proliferation and differentiation of various cell types including macrophages and alveolar epithelial cells. It was shown to have protective effects in bacterial pneumonia of gram-negative (Standiford, et al., 2011) and gram-positive bacteria (Steinwede, et al., 2011).

Neutrophils are the main phagocytic cells that eliminate the bacteria in the airways. After recruitment by the above mentioned mechanisms, they generate pro-inflammatory signals, such as IFNγ (Yamada, et al., 2011), IL-18 (Sporri, et al., 2008), and chemokines (Borregaard, et al., 2007). These chemokines induce further recruitment of immune cells. IL-18 activates different cell types including neutrophils and NK cells and further promotes the production of type II IFN (Fortin, et al., 2009; Sporri, et al., 2008). The IFNγ activates neutrophils to enhance phagocytosis and to produce ROS (Yamada, et al., 2011). Apart from eliminating bacteria by phagocytosis, neutrophils contain granules with antimicrobial peptides and enzymes (e.g. neutrophil elastase, GpIIA-PLA2) that are released during infection (Borregaard, et al., 2007; Moraes, et al., 2006). Moreover, they can constrain and kill bacteria by producing neutrophil extracellular traps (NETs). NETs are composed of decondensed chromatin and antimicrobial proteins. They restrict bacterial spreading and provide a scaffold to enhance the local concentration of antimicrobial proteins (Papayannopoulos and Zychlinsky, 2009). However, some bacteria, such as *S. pneumoniae*, can counteract entrapment by NETs by expressing endonucleases (Beiter, et al., 2006).

Additionally to shaping the innate immune response, DCs migrate to the peripheral lymph nodes and activate T cells, which in turn can stimulate B cells. This leads to the production of antibodies by B cells and to anti-microbial T cell responses. In general, T cells are divided into several subsets including T helper cells, cytotoxic T cells, and natural killer T cells. Together they shape the immune response in the lung. Both $CD8^+$ T cells and Th1/Th17 cells have been shown to contribute to the host defense in bacterial pneumonia (Bai, et al., 2009; Olliver, et al., 2011; Weber, et al., 2011). Th17 cells, however, are most important for fighting pneumonia caused by extracellular bacteria.

In order to avoid excessive lung injury elicited by the antimicrobial mechanisms during the immune response, anti-inflammatory factors and pathways are activated. Negative feedback mechanisms are important since the damage produced by the defense mechanisms can be as lethal as the infection itself. These mechanisms include the production of anti-inflammatory cytokines, such as IL-10, and of lipid molecules including lipoxins, resolvins, and protectins (Carlo and Levy, 2010; Mizgerd, 2008; Seki, et al., 2010).

In order to achieve an optimal immune response to bacterial infection in the lung, a fine balance between the innate immune system, the adaptive immune system, and tissue-preserving anti-inflammatory pathways is required.

1.4 Aim of this study

Type I IFNs are important regulators of the immune system. They induce the expression of hundreds of bona-fide ISGs, and have been shown to regulate (positively or negatively) the production of mediators of the innate and the adaptive immune response. The role of type I IFNs in bacterial infections, however, has only begun to be characterized during the last years. While IFNα/β was shown to have detrimental effects in infections with *L. monocytogenes* and *M. tuberculosis* (O'Connell, et al., 2004; Stanley, et al., 2007), they had a protective role in infections with *L. pneumophila* and group B streptococcus (Lippmann, et al., 2011; Mancuso, et al., 2007). However, in *S. pneumoniae* infection, neither the pathway of type I IFN induction, nor their role in primary pneumococcal pneumonia have been investigated.

The aims of this study were: (I) to investigate the bacterial requirements necessary for a type I IFN response; (II) to characterize the cellular pathways leading to the induction of type I IFNs after pneumococcal infection; (III) to analyze the impact of autocrine and paracrine type I IFNs on macrophages and neighboring epithelial cells; and (IV) to investigate the function of type I IFNs in pneumococcal pneumonia in mice.

This study will contribute to the characterization of a novel pathway activated by *S. pneumoniae*, which is a part of the innate immune response. The investigation of its effects on cytokines and chemokines and of its role during pneumococcal pneumonia *in vivo* will contribute to a detailed understanding of the innate immune response. This will hopefully foster the development of new therapeutic strategies in order to fight pulmonary infections of *S. pneumoniae* in the future.

2. Results

2.1 *Streptococcus pneumoniae* infection leads to the induction of type I interferons dependent on the virulence factor pneumolysin

First, I investigated if *S. pneumoniae* induces type I IFNs in macrophages and what bacterial factors are involved in this induction. The infection of murine bone marrow-derived macrophages (BMMs) with a *S. pneumoniae* serotype 2 strain (D39) led to an induction of IFNβ on both mRNA and protein levels (Fig. 3A, B). A strain deficient for the capsule (D39 Δ*cps*) was also fully capable of inducing type I IFNs and even showed slightly enhanced IFNβ mRNA levels. However, the IFNβ induction was dependent on the pore-forming toxin pneumolysin (PLY), since PLY-deficient strains (D39 Δ*ply*, D39 Δ*cps*Δ*ply*) did not cause an induction of IFNβ. The induction of type I IFNs after pneumococcal infection was also detected in human alveolar macrophages (Fig. 3C) and peripheral blood mononuclear cells (PBMCs; data not shown), but not in the human alveolar epithelial cell line A549 (Fig. 3D) or in primary murine lung alveolar epithelial cells (see Fig. 11C).

This type I IFN induction was neither strain- nor serotype-specific, since infection with a serotype 4 strain derived from a clinical isolate (TIGR4) also led to a significant increase of IFNβ production in BMMs (Fig. 3E). However, the magnitude of induction was much less compared to the D39 wild-type strain. In contrast, the capsule-deficient TIGR4 strain (TIGR4 Δ*cps*) induced comparable IFNβ levels to D39. This induction was also dependent on PLY since the TIGR4 Δ*cps*Δ*ply* strain showed an abrogated induction of type I IFNs (Fig. 3F).

Considering that *S. pneumoniae* stimulated the production of type I IFNs in different phagocytes but not in lung epithelial cells, which are not actively phagocytosing, I hypothesized that the different magnitudes of IFNβ induction by D39 and TIGR4 strains might depend on an unequal phagocytic uptake of the pneumococci. Indeed, the amount of type I IFN mRNA levels correlated with the amount of phagocytosed *S. pneumoniae*. TIGR4 wild-type pneumococci were only weakly phagocytosed by BMMs as opposed to D39 wild-type (Fig. 3G). Capsule-deficient strains were always more strongly ingested than their wild-type counterparts, which also correlated with an enhanced type I IFN induction (Fig. 3A, F).

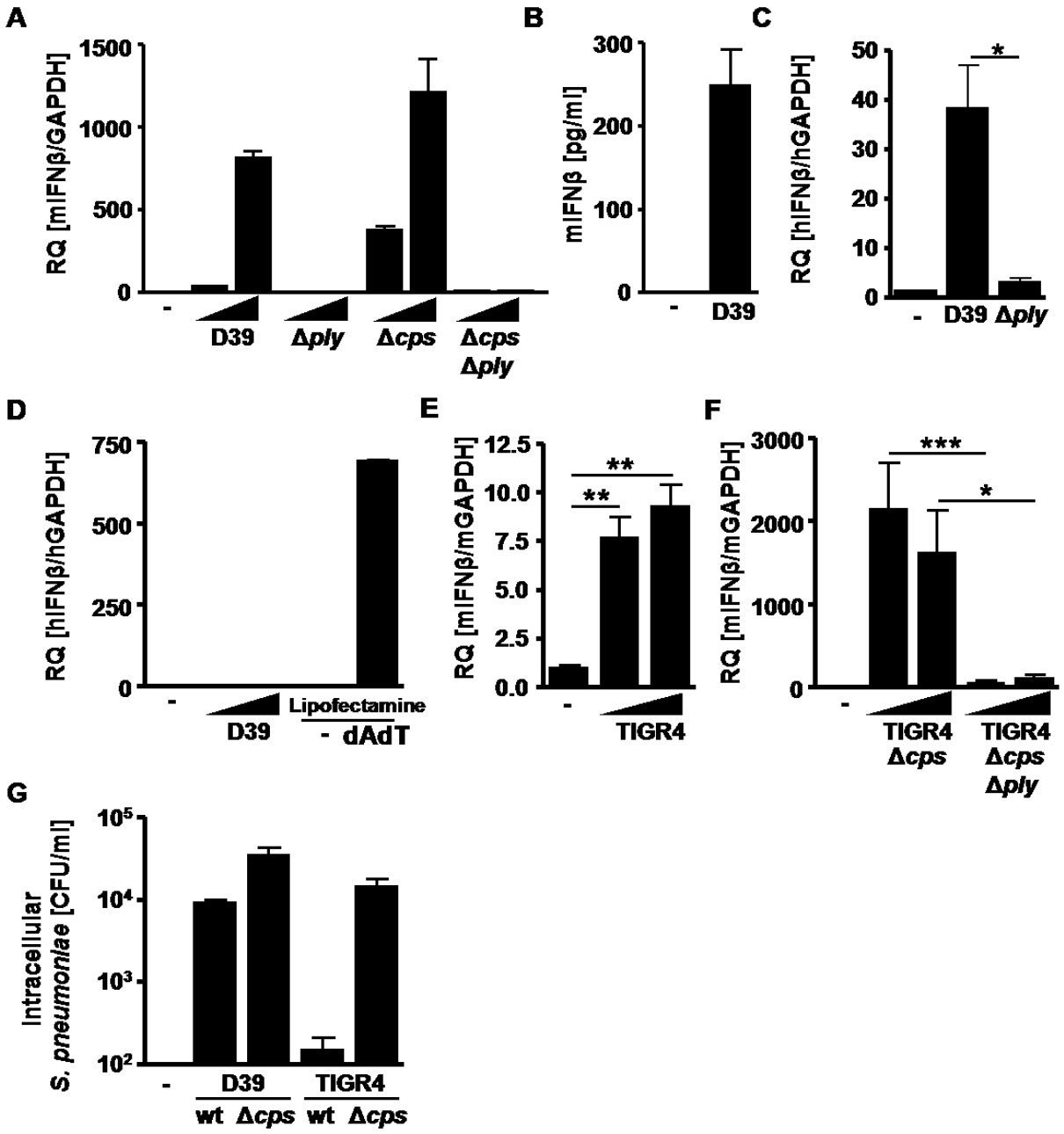

Fig. 3: Type I IFNs are induced in macrophages after infection with *S. pneumoniae* in macrophages dependent on PLY. (A) C57BL/6 wild-type BMMs were infected with D39 wild-type, Δ*ply*, Δ*cps*, or Δ*cps*Δ*ply* pneumococci for 6h with a MOI 0.025 or 2.5. (B) Wild-type BMMs were infected with D39 wild-type (MOI 0.025) for 18 h. (C) Human alveolar macrophages were infected with D39 wild-type or Δ*ply* (MOI 2.5) for 6h. (D) Human broncho-alveolar epithelial cells (A549) were either infected with D39 wild-type (MOI 0.025 or 2.5) or transfected with 0.25 µg poly dA:dT using Lipofectamine 2000 for 6 h. (E) Wild-type BMMs were infected with TIGR4 wild-type pneumococci (MOI 0.025 or 2.5) for 6h. (F) Wild-type BMMs were infected with TIGR4 Δ*cps* or TIGR4Δ*cps*Δ*ply* (MOI 0.025 or 2.5) for 6h. (G) BMMs from C57BL/6 mice were infected with either D39 or TIGR4 (wild-type or Δ*cps*, MOI 2.5) for 1h. Internalized bacteria were determined as described. mIFNβ mRNA levels were determined by quantitative RT-PCR. IFNβ in the supernatant was quantified by ELISA. Experiments were performed twice (B, G) or at least three times (A, C-F) in duplicates (A-D) or triplicates (E-G). Figures show the mean + SEM of either a representative experiment (A, B, D) or the combined data (C, E-G). Where possible, statistical analysis was performed as described. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Because the expression of PLY was essential for the type I IFN induction by both D39 and TIGR4 pneumococci, I tested if PLY alone would be sufficient to trigger the IFNβ induction. The incubation of human PBMCs with purified recombinant PLY did not lead to the induction of type I IFNs in contrast to poly I:C, which was used as a positive control (Fig. 4A). The purified PLY was biologically active, since it was fully capable of eliciting the release of IL-1β from the cells (Fig. 4B) as previously described (Witzenrath, et al., 2011). Thus PLY is necessary, but not sufficient for the type I IFN induction in response to *S. pneumoniae* infection.

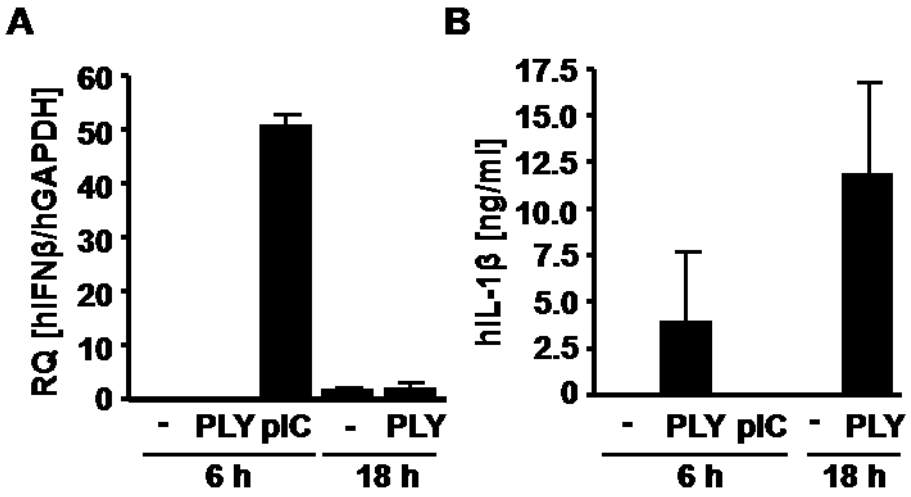

Fig. 4: Purified recombinant PLY is not sufficient to induce type I IFNs in macrophages. Human PBMCs were stimulated with PLY (2 µg/ml) or poly I:C (pIC, 0.5 µg/ml) for 16 h. hIFNβ mRNA levels were determined by quantitative RT-PCR. IL-1β in the supernatant was quantified by ELISA. Data shown are mean + SEM of a representative of three independent experiments carried out in duplicates.

2.2 The type I IFN response to *Streptococcus pneumoniae* is dependent on bacterial uptake and endosomal acidification

To further characterize the requirements for a type I IFN induction, BMMs were incubated with inhibitors for actin polymerization (Fig. 5A, C) or endosomal acidification (Fig. 5B, D). Inhibition of action polymerization with cytochalasin D renders the cell unable to phagocytose and abrogated the type I IFN induction after infection with D39 wild-type (Fig. 5A) or TIGR4 Δ*cps* pneumococci (Fig. 5C). These results are in agreement with the data shown above indicating that the type I IFN response occurs only in cells capable of phagocytosis (Fig. 3A, D, G). Additionally to the phagocytic uptake of *S. pneumoniae*, the acidification of the endosomal compartment is also important in the induction of type I IFNs. Pre-incubation of BMMs with the endosomal acidification inhibitors bafilomycin A1, chloroquine, or ammonium chloride, reduced the IFNβ induction after pneumococcal infection with D39 wild-type (Fig 5B) or TIGR4 Δ*cps* (Fig 5D) strains. Thus, the phagocytic uptake as well as the endosomal acidification are important steps in the initiation of the type I IFN responses to *S. pneumoniae* infection.

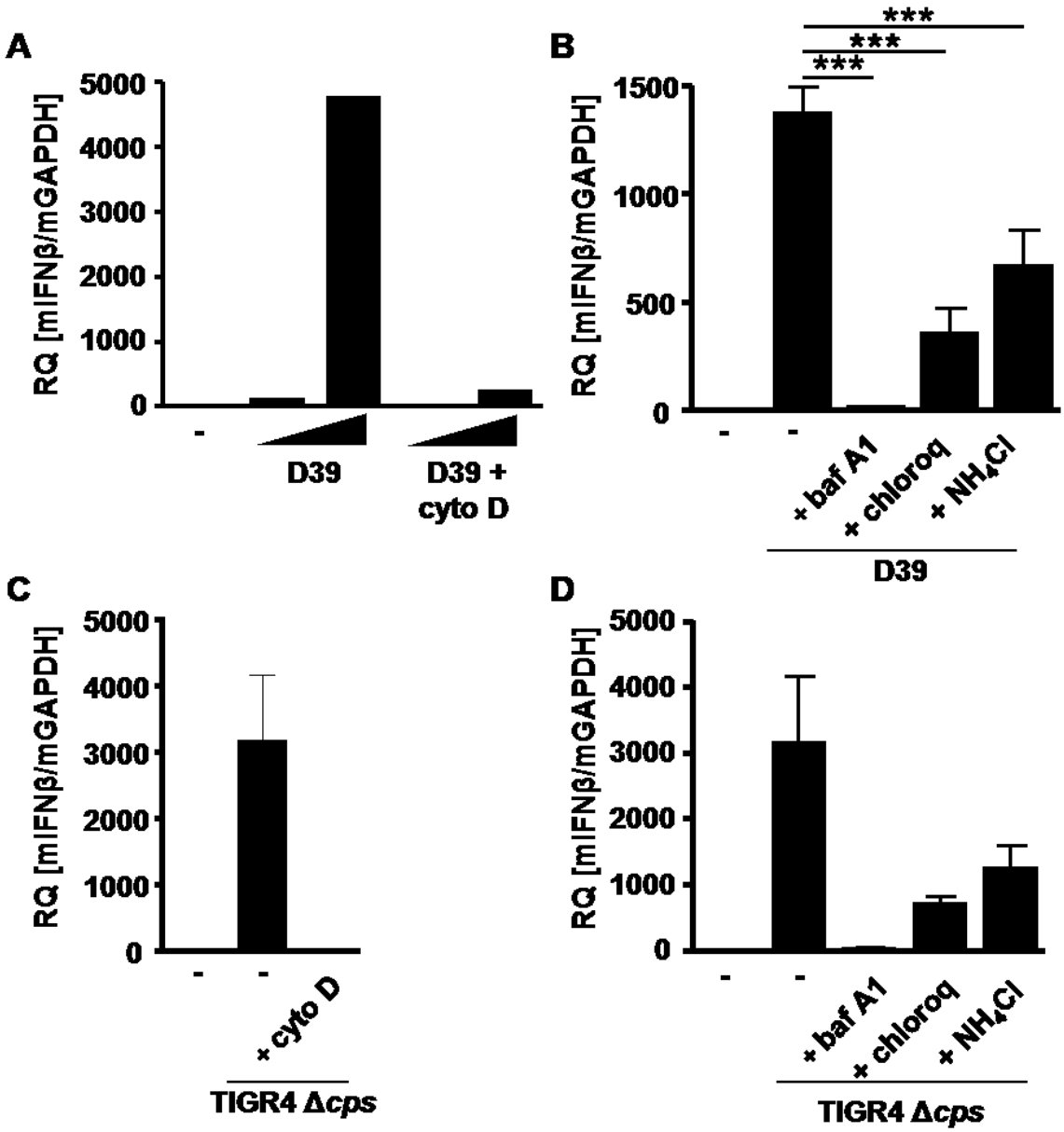

Fig. 5: The type I IFN response is dependent on the phagocytic uptake of the bacteria as well as on the acidification of the endosome. (A, C) C57BL/6 wild-type BMMs were pre-treated with 2 µM Cytochalasin D for 30 min before infection with (A) D39 wild-type (MOI 0.025 or 2.5, 6 h) or (C) TIGR4 Δ*cps* (MOI 2.5, 6 h). (B, D) Wild-type BMMs were stimulated with 200 nM bafilomycin A1, 50 µM chloroquine, or 5 mM ammonium chloride for 30 min and then infected with (C) D39 wild-type (MOI 2.5, 6 h) or (D) TIGR4 Δ*cps* (MOI 2.5, 6 h). mIFNβ mRNA levels were determined by quantitative RT-PCR. Data shown are representatives of two (C, D) or three (A) independent experiments or as mean ±SEM of three independent experiments (B) carried out in duplicates (A, B) or triplicates (C, D). Where possible, statistical analysis was performed as described. *** = $p < 0.001$.

2.3 Type I IFN responses to pneumococcal infection appear to be dependent on intracellular recognition of bacterial DNA

Since PLY was required, but not sufficient for the induction of type I IFNs, other pneumococcal PAMPs must exist that contribute to the activation of downstream signaling pathways. Because the uptake of the bacteria into the cell was required for the IFNα/β response (see Fig 3 and 5), I produced *S. pneumoniae* extracts and transfected them into BMMs.

This transfection caused the induction of IFNβ in these cells (Fig. 6A), which indicates that some pneumococcal components are generally capable of inducing type I IFNs upon delivery into the cytosol. Moreover, extracts from both D39 wild-type and D39 Δ*ply* strains elicited similar amounts of IFNβ induction after transfection into macrophages (Fig. 6A). Interestingly, digestion of D39 wild-type extracts with DNase, RNaseA, RNaseH, or proteinase K before transfection demonstrates that only the digestion with DNase led to an abrogation of the IFNβ induction (Fig. 6B). Moreover, the transfection of purified *S. pneumoniae* DNA into BMMs also elicited an increase in IFNβ mRNA levels (Fig. 6C).

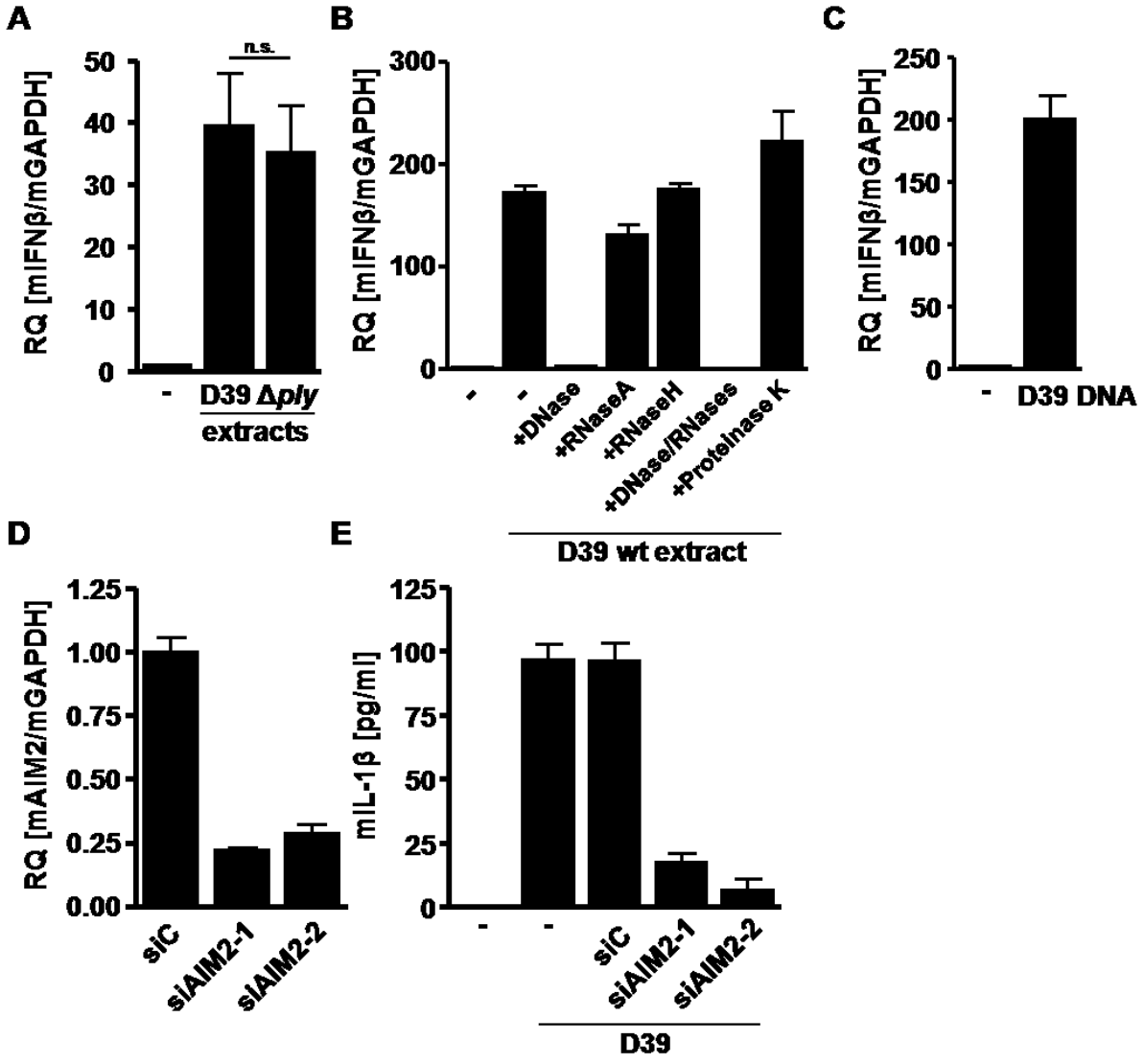

Fig. 6: The induction of type I IFNs appears to be dependent on the intracellular recognition of DNA. (A, B) Extracts from (A) D39 wild-type and Δ*ply* strains or from (B) D39 wild-type strain digested with DNase, RNases, or proteinase were transfected into C57BL/6 wild-type BMMs for 6 h. (C) *S. pneumoniae* DNA (0.25 µg per well) was transfected into wild-type BMMs. (D, E) Wild-type BMMs were transfected with siRNAs against AIM2 or with an unspecific control siRNA (siC) for 48 h before infection with D39 wild-type (MOI 0.025) for 16 h. mIFNβ (A-C) or mAIM2 (D) mRNA levels were determined by quantitative RT-PCR. mIL-1β in the supernatant was measured by ELISA. Data shown are representatives of two (A, D) or three (B, C, E) experiments carried out in duplicates (A-D) or triplicates (E).

These results indicate that pneumococcal DNA is generally capable of inducing type I IFNs in BMMs. However, the delivery of DNA into the cytosol via transfection is artificial and might not reflect the physiological situation during an infection. Thus, to prove that DNA is indeed present in the host cell cytosol during *S. pneumoniae* infection, I examined if AIM2, a well-characterized cytosolic DNA sensor is activated by pneumococci. AIM2 belongs to the family of PYHIN proteins and forms an inflammasome after sensing e.g. microbial DNA in the cytosol of host cells (see above). This inflammasome processes pro-IL-1β into its mature form. Thus, the examination if the IL-1β production during pneumococcal infection is dependent on AIM2 indicates whether DNA is generally present in the cytosol during infection with *S. pneumoniae*. Indeed, the knockdown of AIM2 by two different siRNAs (Fig. 6D) led to a strong decrease in the IL-1β production after pneumococcal infection (Fig. 6E). This indicates that the AIM2 inflammasome is involved in sensing *S. pneumoniae* and that DNA – probably of pneumococcal origin – is present in the cytosol during infection. Taken together, recognition of cytosolic DNA might be the PAMP that together with PLY is required for the induction of type I IFNs in *S. pneumoniae*-infected macrophages.

2.4 Type I IFN induction by *Streptococcus pneumoniae* is dependent on STING, MyD88, and IRF3

In order to identify the cellular signaling pathway that is involved in the induction of the type I IFN response, BMMs from several knockout mice (C57BL/6 background) were infected and the IFNβ mRNA levels in these cells were compared to the ones in wild-type cells. BMMs deficient in TLR2/3/4/7/9 or in the TLR3/4 adaptor TRIF did not show an impaired IFNβ induction compared to their wild-type counterparts (Fig. 7A, D). However, agonists for the respective TLRs failed to induce a response in the knockout cells (Fig. 7B, C, E, and data not shown). Surprisingly, BMMs deficient in the TLR adaptor MyD88 had a significantly reduced type I IFN response after infection with *S. pneumoniae* compared to the wild-type cells (Fig. 7F). The knockout was functionally confirmed by showing the absent response of the MyD88-/- BMMs to the TLR2/4 agonists MALP2 and LPS (Fig. 7G). Thus, MyD88 – but not TLR2/3/4/7/9 or TRIF – is involved in the type I IFN response after pneumococcal infection.

Next, I tested the involvement of several intracellular pathways that have been shown to be able to contribute to the induction of type I IFNs. BMMs from NOD2-/- mice showed an unaltered IFNβ induction compared to their wild-type counterparts after infection with *S. pneumoniae* (Fig. 8A). The functionality of the knockout was confirmed by detecting an almost absent IL-1β induction in these cells after stimulation with the NOD2 agonist MDP (Fig. 8B). Moreover, MAVS-/- BMMs showed a strong *S. pneumoniae*-induced IFNβ production (Fig. 8C), indicating that both the RNA-sensing RLRs and the DNA-sensing RNA PolIII/RIG-I pathway were not involved in this response. However, the knockout BMMs failed

to respond to the RIG-I agonists polyI:C and pppRNA (Fig. 8D), which confirmed that these cells were deficient in RIG-I signaling. Additionally, the involvement of IRF3, a central transcription factor for the initiation of the type I IFN response, was investigated. BMMs from IRF3-/- mice had a significantly abrogated IFNβ induction after infection with both D39 wild-type (Fig. 8E) and TIGR4 Δ*cps* (Fig. 8F) pneumococci. Thus, IRF3 – but not NOD2 or MAVS-related pathways – are required in the induction of type I IFNs in response to *S. pneumoniae* infection.

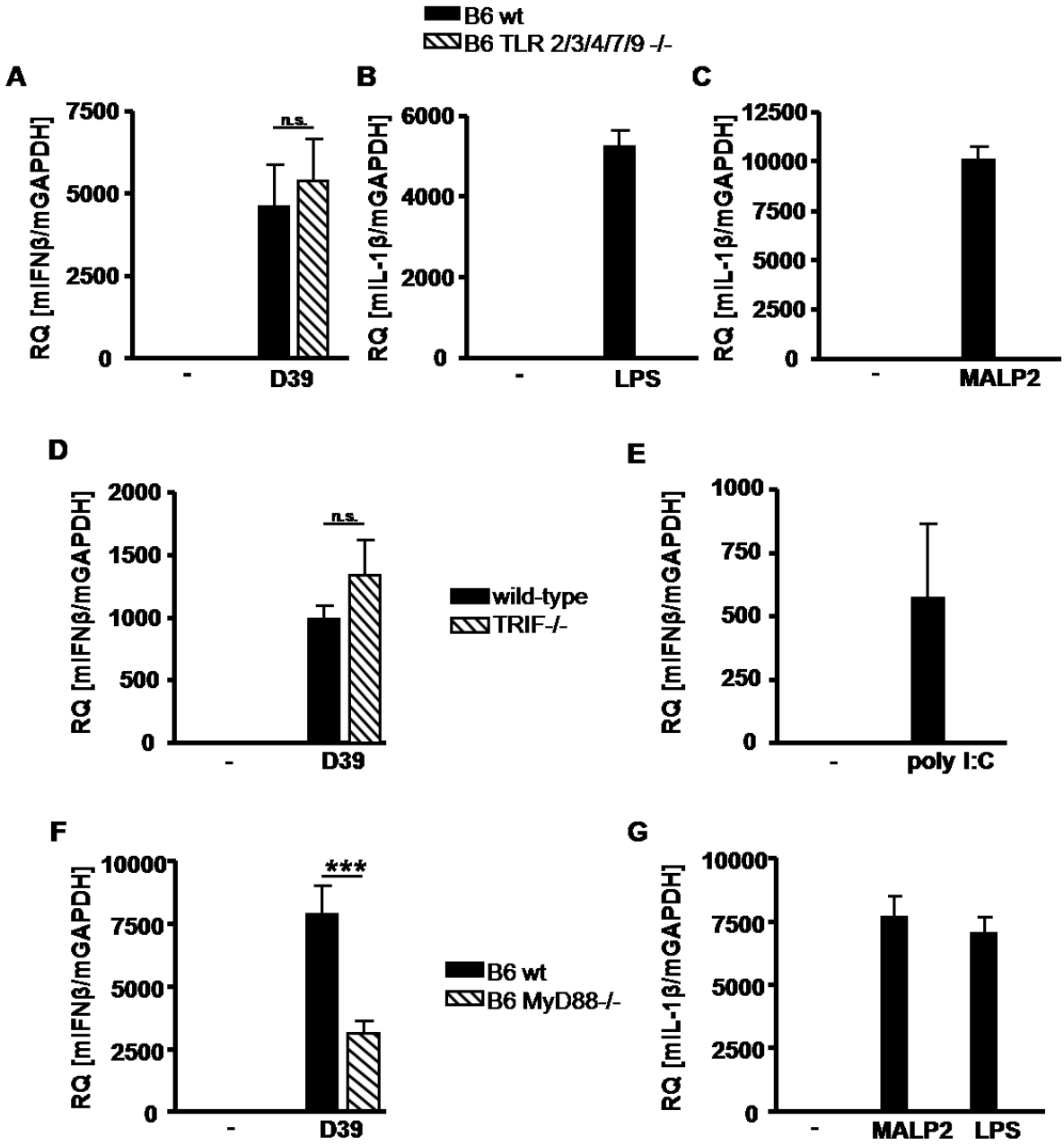

Fig. 7: The type I IFN response after pneumococcal infection is dependent on MyD88. BMMs from C57BL/6 wild-type or the respective knockout mice were infected with D39 wild-type strain (MOI 2.5, 6 h) (A, D, F), or stimulated with the respective PRR agonists for 6 h (B, C, E, G). mRNA levels of mIFNβ and mIL-1β were determined by quantitative RT-PCR. Infection experiments were performed three times in duplicates (A) or triplicates (D, F). Figures show the mean + SEM of either a representative experiment (B, C, E, G) or the combined data (A, D, F). Where possible, statistical analysis was performed as described. *** = p < 0.001.

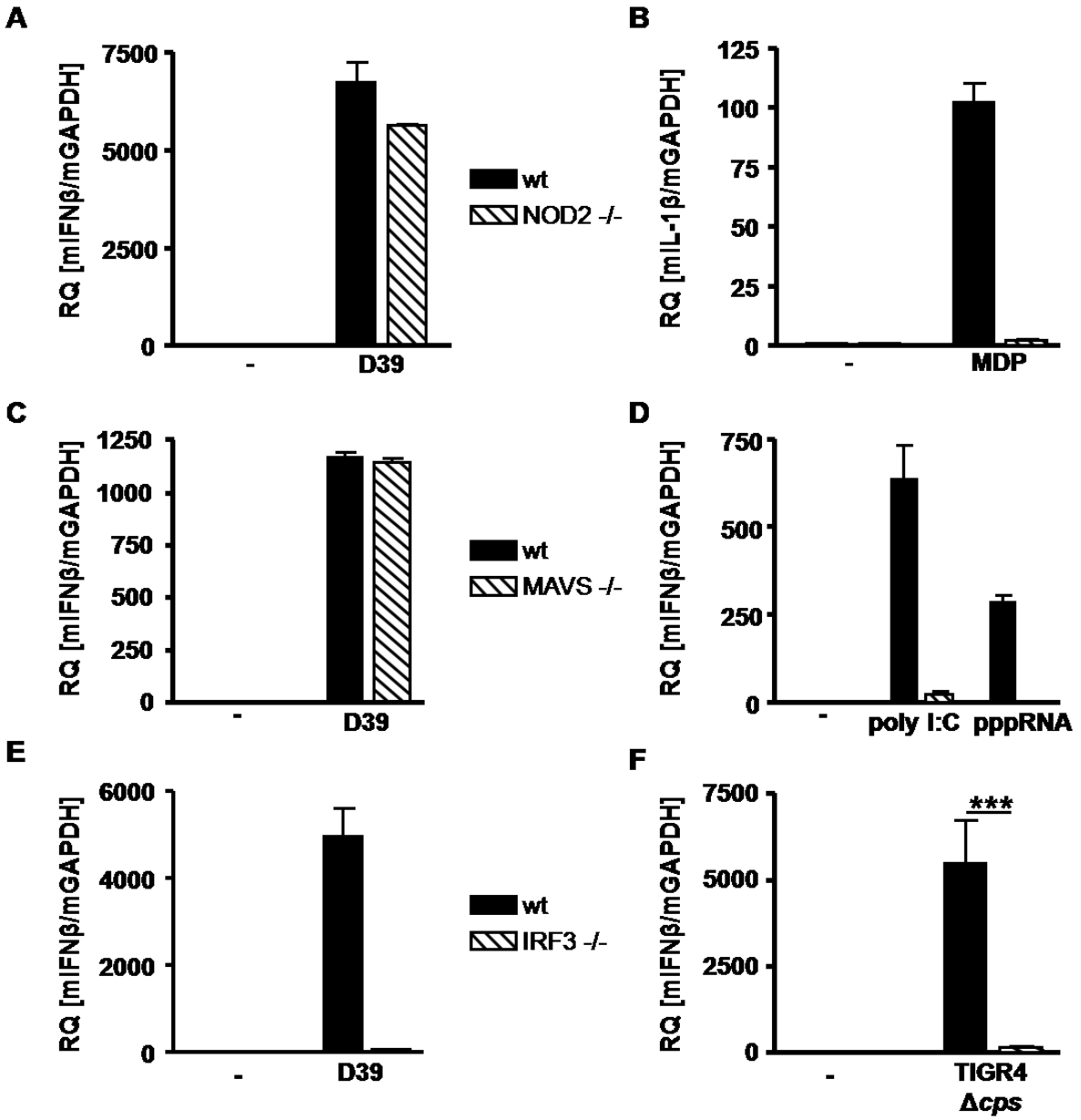

Fig. 8: The type I IFN response after pneumococcal infection is dependent on IRF3. BMMs from C57BL/6 wild-type or the respective knockout mice were infected with D39 wild-type strain (A, C, E), TIGR4 Δ*cps* strain (F) (MOI 2.5, 6 h), or stimulated with the respective PRR agonists for 6 h (B, D). mRNA levels of mIFNβ and mIL-1β were determined by quantitative RT-PCR. Data shown are representatives of at least three experiments carried out in duplicates. Infection experiments were performed three times in duplicates (A, C, E) or triplicates (F). Figures show the mean + SEM of either a representative experiment (A - E) or the combined data (F). Where possible, statistical analysis was performed as described. *** = p < 0.001.

In order to further elucidate cellular proteins involved in the type I IFN response to *S. pneumoniae*, RNA interference (RNAi) experiments were performed. The siRNA-mediated knockdown of STING (Fig. 9A), an adaptor protein for many cytosolic DNA sensors, led to a reduced IFNβ induction after infection with both D39 wild-type (Fig. 9B) and TIGR4 Δ*cps* (Fig. 9C) pneumococci. Since this and earlier results indicate that pneumococcal DNA might be involved in the type I IFN response, I tested the involvement of two PRRs that have been described to detect DNA in the cytosol, DAI and IFI16. Knockdown of either protein with

different specific siRNAs (Fig. 9D, F) did not result in a strong reduction of the type I IFN response after pneumococcal infection (Fig. 9E, G). In the case of DAI, a tendency of reduction was detected. However, this effect seems negligible due to its small magnitude. Thus, STING is involved in the IFNα/β response to *S. pneumoniae*, but the cytosolic DNA-sensing PRRs IFI16 and DAI do not play a non-redundant role in this signaling pathway.

In summary, the adaptor molecules MyD88 and STING as well as the transcription factor IRF3 are involved in the type I IFN response after pneumococcal infection.

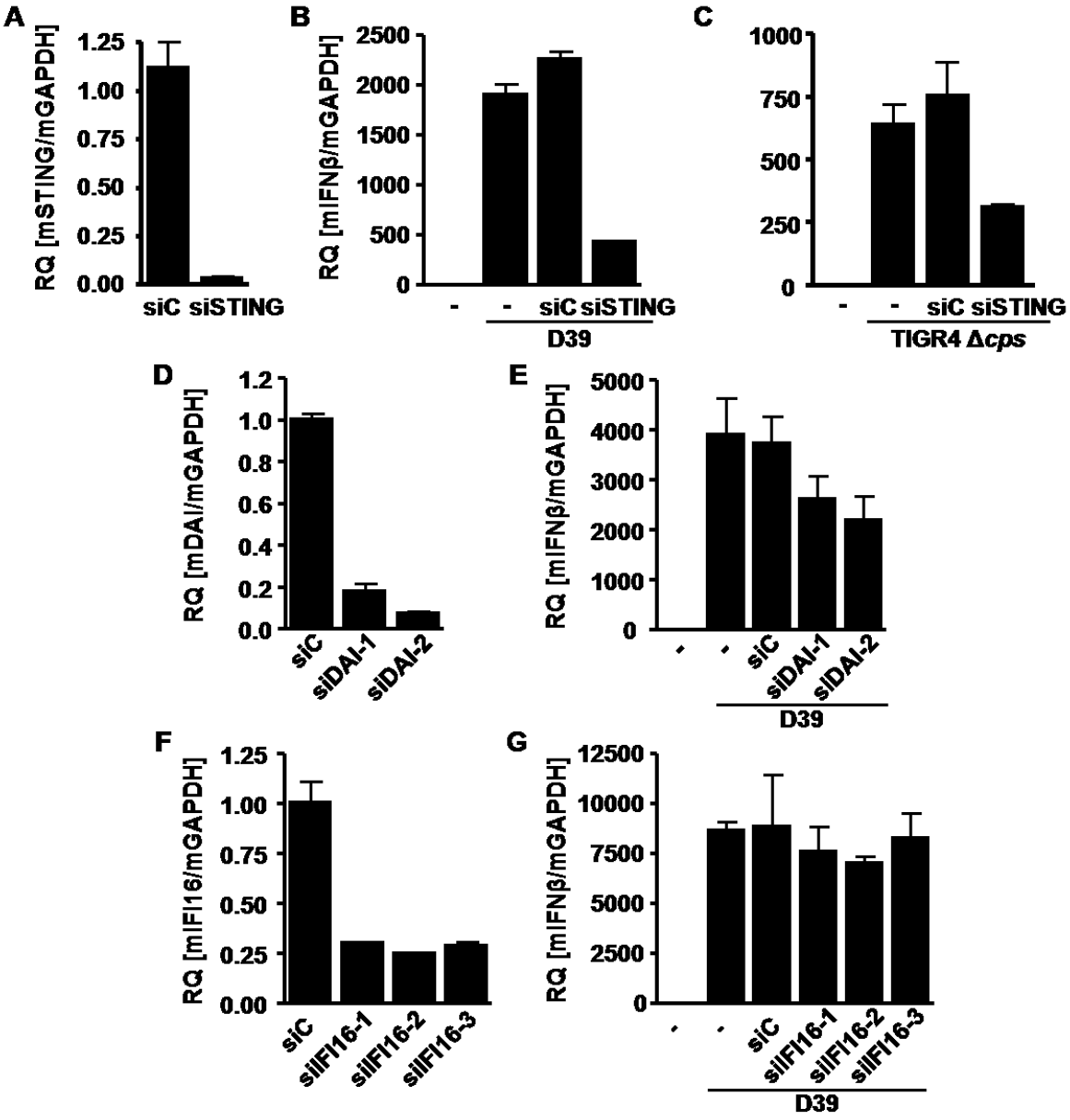

Fig. 9: The type I IFN response after pneumococcal infection is dependent on STING. Wild-type BMMs were transfected with siRNAs specific for STING (A-C), DAI (D, E), IFI16 (F, G) or with unspecific control siRNA (siC) for 48 h prior to infection with D39 wild-type strain (B, E, G) or TIGR4 Δ*cps* strain (C) (MOI 2.5, 6 h). mRNA levels of mIFNβ were determined by quantitative RT-PCR. Experiments were performed at least three times in duplicates. Figures show the mean + SEM of representative experiments.

2.5 Type I IFNs regulate the production of RANTES in macrophages in an autocrine manner

After investigating the pathways that lead to the type I IFN induction after pneumococcal infection, I then focused on the autocrine effects of type I IFNs on *S. pneumoniae*-infected macrophages. I investigated RANTES, a chemokine that was shown to be important in the host defense against *S. pneumoniae* (Palaniappan, et al., 2006). Indeed, type I IFNs induced by pneumococci stimulate the expression of the chemokine RANTES in BMMs on both mRNA and protein levels (Fig. 10A, B). BMMs deficient for the type I IFN receptor (IFNAR) – and thus unresponsive to the secreted type I IFNs – produce significantly reduced RANTES levels after infection with *S. pneumoniae* compared to wild-type BMMs.

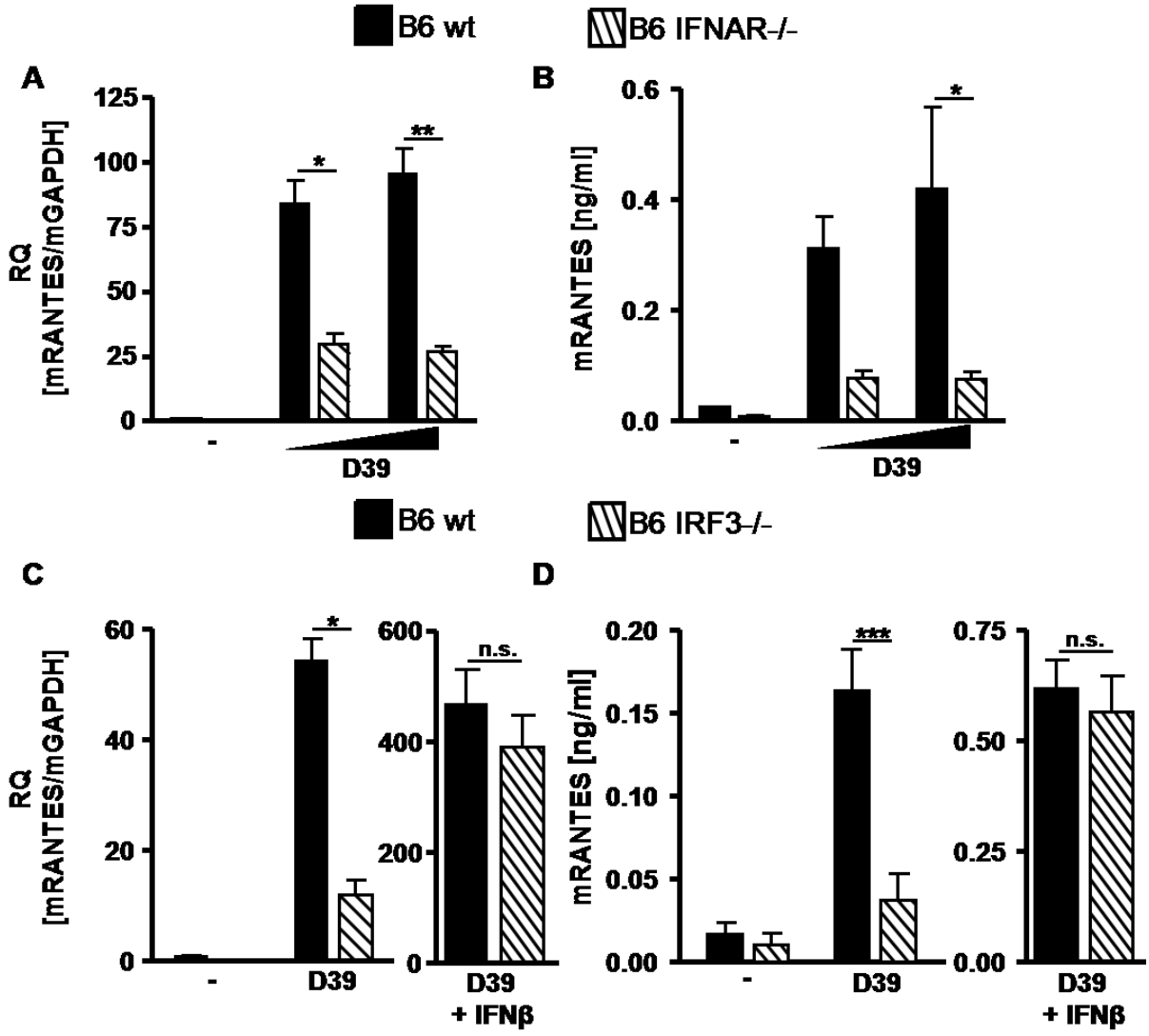

Fig. 10: Type I IFNs regulate the production of RANTES in an autocrine manner. (A, B) BMMs from wild-type or IFNAR-/- mice were infected with D39 wild-type pneumococci (MOI 0.0025 or 0.025) for 16 h. (C, D) BMMs from wild-type or IRF3-/- mice were either infected with D39 wild-type pneumococci alone or stimulated with mIFNβ prior to and during the infection. RANTES mRNA levels were determined by quantitative RT-PCR. Secreted RANTES in the supernatant was quantified by ELISA. Data shown are mean ± SEM of at least three independent experiments carried out in triplicates. Statistical analysis was performed as described. * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

In order to rule out that the difference in RANTES production was owing to a general defect in IFNAR-/- BMMs and not to type I IFN-related signaling, I sought to confirm this result in IRF3 -/- BMMs. As shown earlier, these macrophages were unable to up-regulate the production of type I IFNs after pneumococcal infection (Fig. 8 E, F). In accordance with the finding in IFNAR-/- BMMs, these cells also produced diminished levels of RANTES on the mRNA and protein level compared to their wild-type counterparts (Fig. 10C, D). Importantly, the defect of IRF3-/- cells in RANTES production after infection with *S. pneumoniae* could be rescued and even enhanced by the addition of exogenous IFNβ. Together, the results demonstrate that type I IFNs regulate RANTES production in macrophages in an autocrine manner.

2.6 Type I IFNs produced by alveolar macrophages regulate the immune response of co-cultured alveolar epithelial cells in a paracrine manner

In order to investigate the effects of type I IFNs in a more complex setting and with cells that are physiologically affected in pneumococcal pneumonia, a co-culture model of primary murine alveolar epithelial cells and alveolar macrophages was used (Fig. 11A). When the two cell types were infected separately with pneumococci, IFNβ was induced in alveolar macrophages but not in alveolar epithelial cells (Fig. 11B, C), which corroborates earlier findings. Next, alveolar epithelial cells from wild-type or IFNAR-/- mice were grown on transwell inserts and co-cultured with wild-type alveolar macrophages. Type I IFN produced by the macrophages would then be able to influence the gene expression in the co-cultured epithelial cells (Fig. 11A).

After infection, ISGs such as ISG15, IRF7, and RANTES were induced in the co-cultured alveolar epithelial cells (Fig. 11D-F). Since epithelial cells do not produce type I IFNs themselves after *S. pneumoniae* infection (Fig. 11C), secreted type I IFNs from the macrophages might have positively influenced the expression of these genes in epithelial cells. This assumption is confirmed by the finding that co-cultured epithelial cells lacking the type I IFN receptor IFNAR showed significantly abrogated or reduced mRNA levels of these genes. Furthermore, RANTES concentrations were reduced in the supernatants of infected wild-type macrophages co-cultured with IFNAR-/- alveolar epithelial cells compared to the supernatants of co-cultured wild-type cells (Fig. 11G). This suggests that RANTES is type I IFN-dependently produced by alveolar epithelial cells in addition to the production by alveolar macrophages. Given that alveolar epithelial cells outnumber alveolar macrophages in the lung, the relevance of the type I IFN-dependent induction in epithelial cells is evident. Thus, type I IFNs produced by macrophages paracrinely regulate and shape the innate immune response in epithelial cells during infection with *S. pneumoniae*.

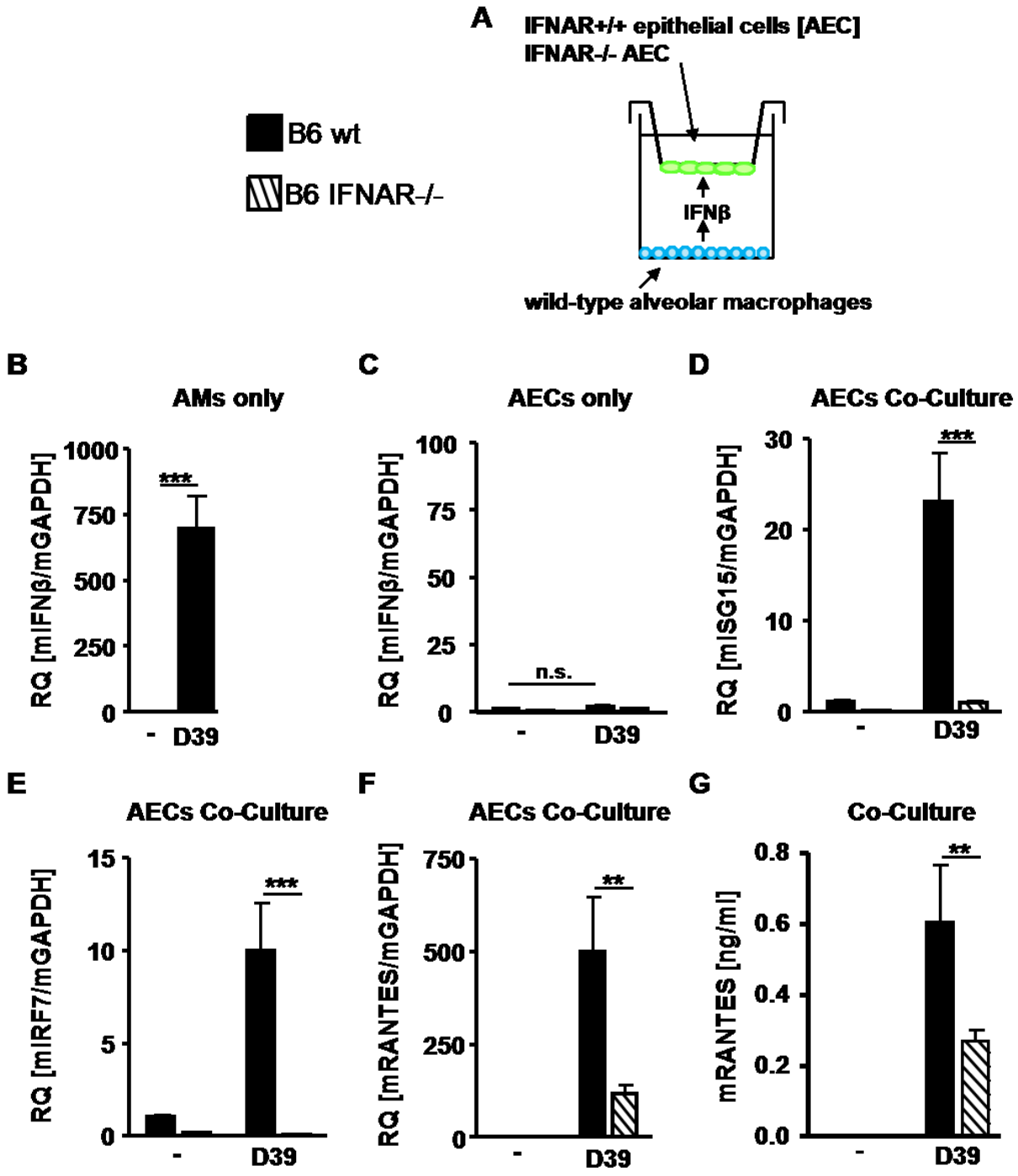

Fig. 11: Type I IFNs secreted by alveolar macrophages regulate the production of ISGs in co-cultured alveolar epithelial cells. (A) Schematic picture of the co-culture model. (B) Primary alveolar macrophages from C57BL/6 wild-type mice were infected with D39 (MOI 2.5) for 6 h. (C) Primary alveolar epithelial cells from C57BL/6 wild-type or IFNAR-/- mice were infected with D39 wild-type strain (MOI 2.5) for 6 h. (D-G) Alveolar macrophages from C57BL/6 wild-type mice were co-cultured with alveolar epithelial cells from wild-type or IFNAR-/- mice and subsequently infected with D39 wild-type strain (MOI 0.025) for 16 h. After infection, the epithelial cells were separated from the macrophages and mRNA expression was analyzed. mRNA expression of IFNβ, ISG15, IRF7, and RANTES was analyzed by quantitative RT-PCR. Secretion of RANTES into the supernatant of co-cultured cells was analyzed by ELISA. Data shown are mean ± SEM of at least three independent experiments carried out in duplicates. Statistical analysis was performed as described; ** = $p < 0.01$, *** = $p < 0.001$.

2.7 Effect of type I and type II IFNs on the progression of pneumococcal pneumonia

After analyzing the pathway leading to type I IFN production and characterizing the effects of type I IFNs on the regulation of ISGs and RANTES *in vitro*, I studied the role of type I IFNs in an *in vivo* model of pneumococcal pneumonia. For this, IFNAR deficient mice were used, which are unable to respond to type I IFNs. Additionally, IFNGR knockout mice and IFNAR/IFNGR knockout mice were used in order to compare the effects of type I and II IFNs during pneumococcal pneumonia, and to examine potential synergistic effects of both IFN families. C57BL/6 mice (wild-type, IFNAR-/-, IFNGR-/-, IFNAR/IFNGR-/-) were infected intranasally with $1x10^6$ cfu *S. pneumoniae* (NCTC7978; serotype 3). After 12 h or 48 h, the mice were sacrificed with subsequent analysis of the infection parameters and the immune response. The bacterial load in the broncho-alveolar lavage (BAL) of mice 12 h post infection (p.i.) remained equally high with no significant differences between the four genotypes (Fig. 12A).

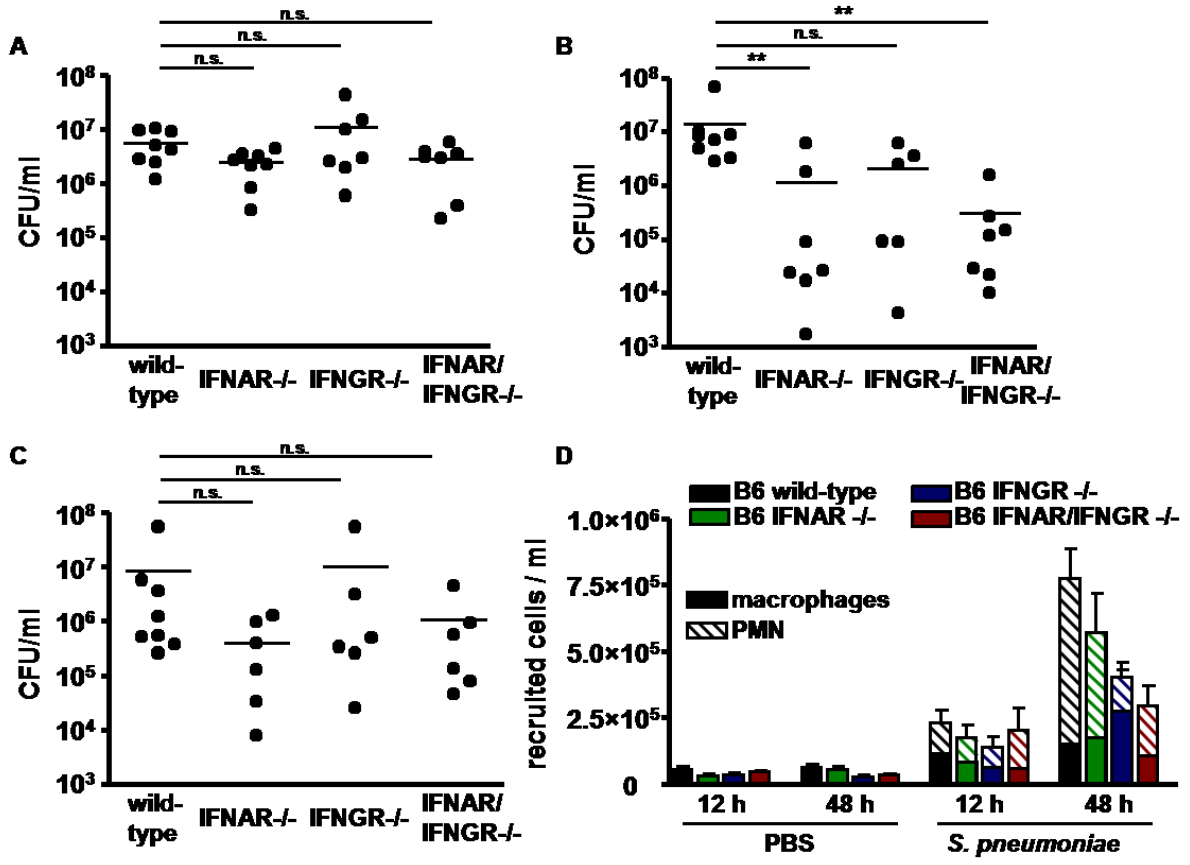

Fig. 12: Type I IFNs negatively affect the bacterial replication but not the cell recruitment in the lung during pneumococcal pneumonia. C57BL/6 mice (wild-type, IFNAR-/-, IFNGR-/-, IFNAR/IFNGR-/-) were intranasally treated with PBS or infected with *S. pneumoniae* ($1x10^6$ cfu, NCTC7978; serotype 3). (A, B) CFU in the BAL were analyzed at (A) 12 h or (B) 48 h p.i. (C) CFU in the blood were analyzed at 48 h p.i. (D) Cells in the BAL were counted and cell types were analyzed with the CytoSpin method. Sample size: PBS-treated: n = 3, infected: n = 6-8. Data are shown as mean ± SEM. Statistical analysis was performed as described; ** = $p < 0.01$.

However, the mean bacterial load in IFNAR-/- and IFNAR/IFNGR-/- mice tended to be slightly lower compared to wild-type and IFNGR-/- mice. In the blood, no pneumococci could be detected at this time point (data not shown). At 48 h p.i., the bacterial numbers in both IFNAR-/- and IFNAR/IFNGR-/- mice were significantly reduced compared to the wild-type mice (Fig. 12B). Moreover, there was a trend of a diminished bacterial burden in the BAL when comparing wild-type and IFNGR-/- mice as well as IFNGR-/- and IFNAR/IFNGR-/- mice. This trend is, however, not statistically significant. At this time point, all mice showed positive blood cultures, demonstrating a spread of the pneumococci from the lung into the whole organism (Fig. 12C). A trend to reduced bacterial numbers in the blood of IFNAR-/- and IFNAR/IFNGR-/- mice could be observed that was, however, not statistically significant. These results show that type I IFNs negatively affect the clearance of *S. pneumoniae* from the lung. Moreover, type II IFN may also have a weak negative effect on the bacterial clearance as well, but not as pronounced as the effect of type I IFNs.

The analysis of the cells in the BAL with the CytoSpin method revealed that all PBS-treated mice only have low numbers of macrophages present in the lavage fluid (Fig. 12D) The genotype did not influence the amount of cells present in the BAL of these uninfected mice. After infection, the amount of both polymorphonuclear leukocytes (PMNs) and, to some extent, of macrophages increased in a time-dependent manner. The recruitment of PMNs was similar in all mouse strains 12 h after infection. At 48 h p.i. the number of PMNs was slightly reduced in IFNAR-/- mice and more strongly diminished in IFNGR-/- and IFNAR/IFNGR-/- mice. The differences in the cell recruitment into the lung do not appear to be responsible for the enhanced clearance of the bacteria in these animals, since an increased recruitment of the cells did not correlate with an augmented clearance in these animals. In contrast, the enhanced clearance of bacteria in the lung of the mice at 48h p.i could have influenced the diminished recruitment of PMNs since a reduced bacterial burden might necessitate a weaker immune response. However, this does not explain, why IFNAR-/- mice with equally reduced bacterial numbers in the BAL to IFNAR/IFNGR-/- mice did not show an equally strong reduction in the recruited cells. Overall, it appears unlikely that effects of the type I IFNs or type II IFN on PMN recruitment into the lungs are responsible for the different bacterial clearance in the lung of the mice strains examined

Analysing the body weight of the mice during the course of infection revealed that all mice had an initial loss of body weight by about 5% of their initial weight after intranasal inoculation with PBS or *S. pneumoniae* (Fig. 13A, B). This was likely due to the lack of food uptake during and after the narcosis at the time of infection. Moreover, while PBS-treated mice recovered fast and started gaining weight in the following time, infected mice (wild-type, IFNAR-/-, IFNGR-/-) continuously lost weight until 48 h p.i. (Fig. 13B). The weight loss was,

however, attenuated in IFNAR/IFNGR-/- mice with significantly less reduction of the body weight compared to wild-type mice.

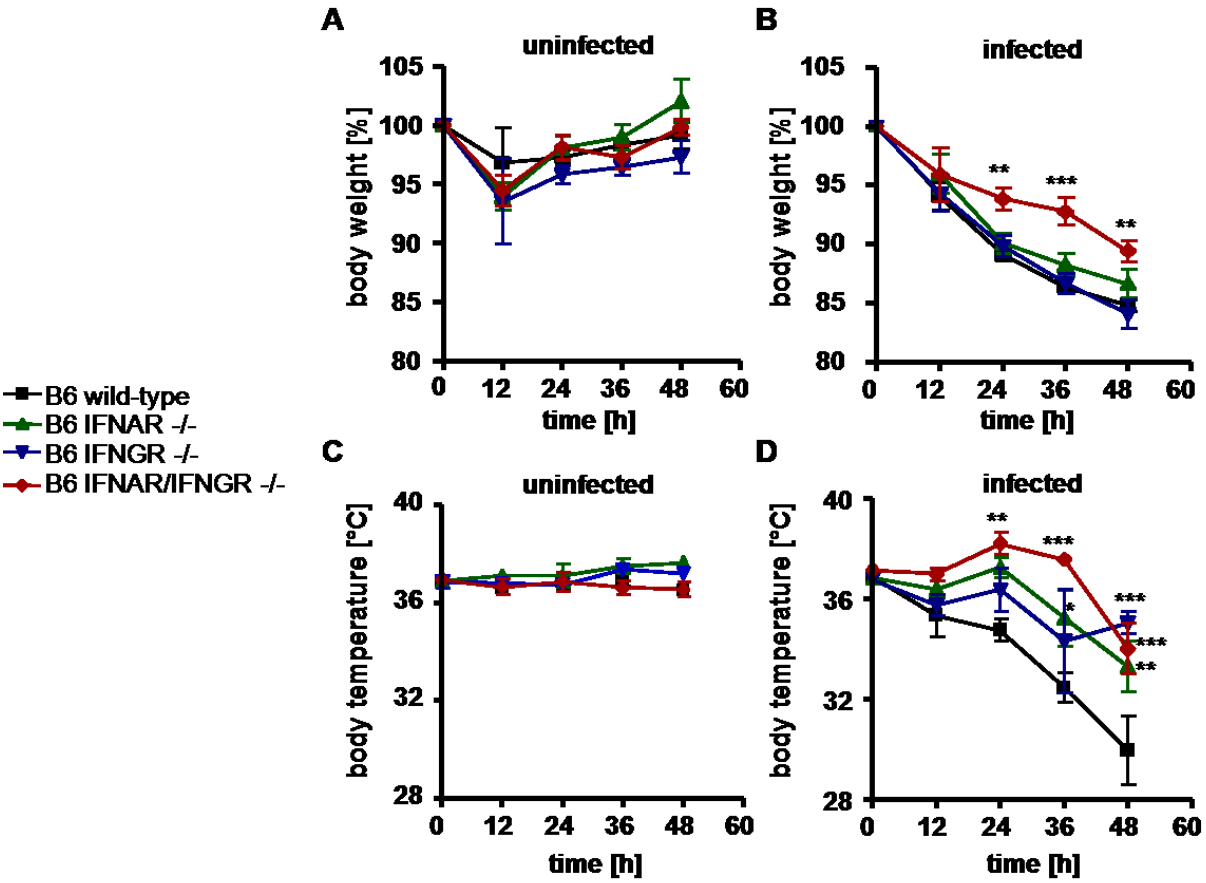

Fig. 13: Absence of type I and type II IFN signaling cooperatively reduces the loss of body weight and body temperature in mice with pneumococcal pneumoniae. C57BL/6 mice (wild-type, IFNAR-/-, IFNGR-/-, IFNAR/IFNGR-/-) were intranasally treated with PBS or infected with *S. pneumoniae* ($1x10^6$ cfu, NCTC7978; serotype 3). (A, B) Body weight and (C, D) body temperature of the mice was measured every 12 h after PBS-treatment or infection. Sample size: PBS-treated: n = 3, infected: n = 6-8. Data are shown as mean ± SEM. Statistical analysis was performed as described; ** = $p < 0.01$, *** = $p < 0.001$.

Another clinical parameter, the body temperature, also revealed a difference between the genotypes. While all PBS-treated mice kept a constant body temperature throughout the experiment (Fig. 13C), infected wild-type mice were unable to maintain their body temperature at the initial level and it kept dropping up to ~30°C at 48 h p.i. (Fig. 13D). In contrast, both IFNAR-/- and IFNGR-/- mice showed a significantly diminished temperature reduction compared to the wild-type mice at later time points. The IFNAR/IFNGR double deficient mice had an even slightly elevated temperature at 24 h and 36 h p.i. that remained significantly elevated throughout the experiment compared to wild-type mice. Thus, both the type I and type II IFN system negatively influence the sustainment of the body weight and temperature during pneumococcal pneumonia in mice. Taken together, these results suggest that both types of IFNs are somewhat detrimental for the mice during pneumococcal pneumonia. Mice double deficient for type I and II IFN signaling appear to be more resistant to pneumococcal pneumonia.

2.8 Effect of type I and type II IFNs on cytokines in the broncho-alveolar lavage during pneumococcal pneumonia

Since type I IFNs, and to a minor degree type II IFN, seem to negatively influence the bacterial clearance from the lung and the resistance of mice towards pneumococcal pneumonia, the influence of these systems on the secretion of various cytokines and chemokines in the BAL was investigated.

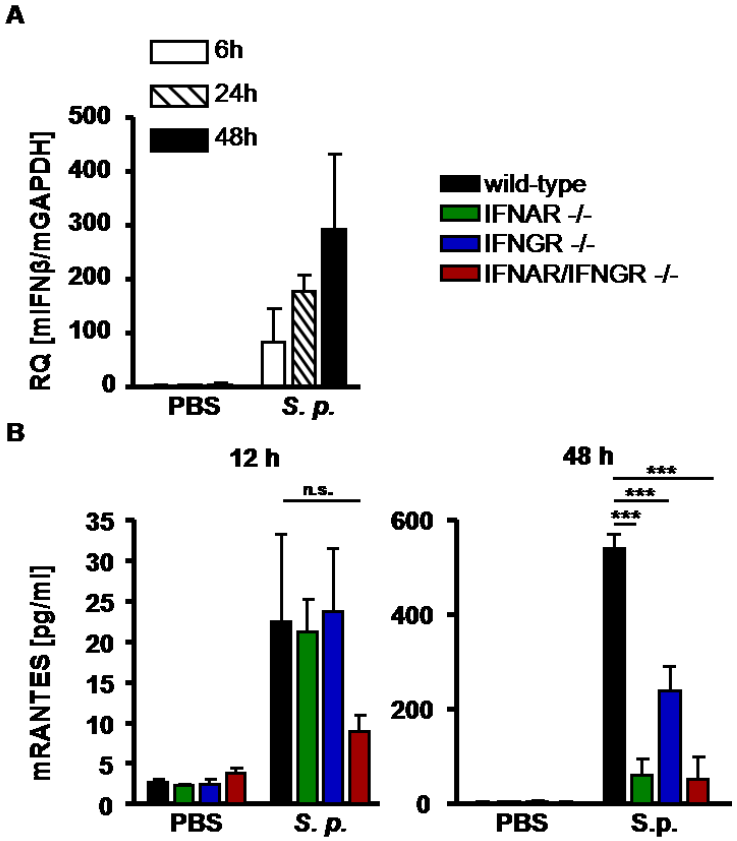

Fig. 14: Production of IFNβ and RANTES in the lung during pneumococcal pneumonia (A) C57BL/6 mice (wild-type) were infected with *S. pneumoniae* (1x10^6 cfu, NCTC7978; serotype 3). After the indicated time-points, the lungs were harvested, RNA was isolated and the expression of mIFNβ was analyzed with qPCR; n = 3 (B) C57BL/6 mice (wild-type, IFNAR-/-, IFNGR-/-, IFNAR/IFNGR-/-) were intranasally treated with PBS or infected with *S. pneumoniae* as described. RANTES concentrations in the BAL were determined by ELISA; PBS-animals: 12 h n = 2; 48 h n = 6; infected animals: n = 5-6. Data shown are mean ±SEM. Statistical analysis was performed as described; *** = p < 0.001.

First, it was confirmed that IFNβ was induced in the lung during pneumococcal pneumonia (Fig. 14A). The respective mRNA was detectable as early as 6 h p.i. and increased over time throughout the experiment. The production of RANTES, as shown *in vitro* in different infection models, was also detected *in vivo* in the BAL of infected mice (Fig. 14B). The overall induction at 12 h p.i. was still very low and a trend to reduced levels of RANTES was only observable in IFNAR/IFNGR-/- mice. However, at 48 h p.i., a sustained production of RANTES in wild-type mice and a slightly reduced production in IFNGR-/- mice could be detected. Both IFNAR-/- and IFNAR/IFNGR-/- mice showed an almost abrogated production of RANTES in the BAL.

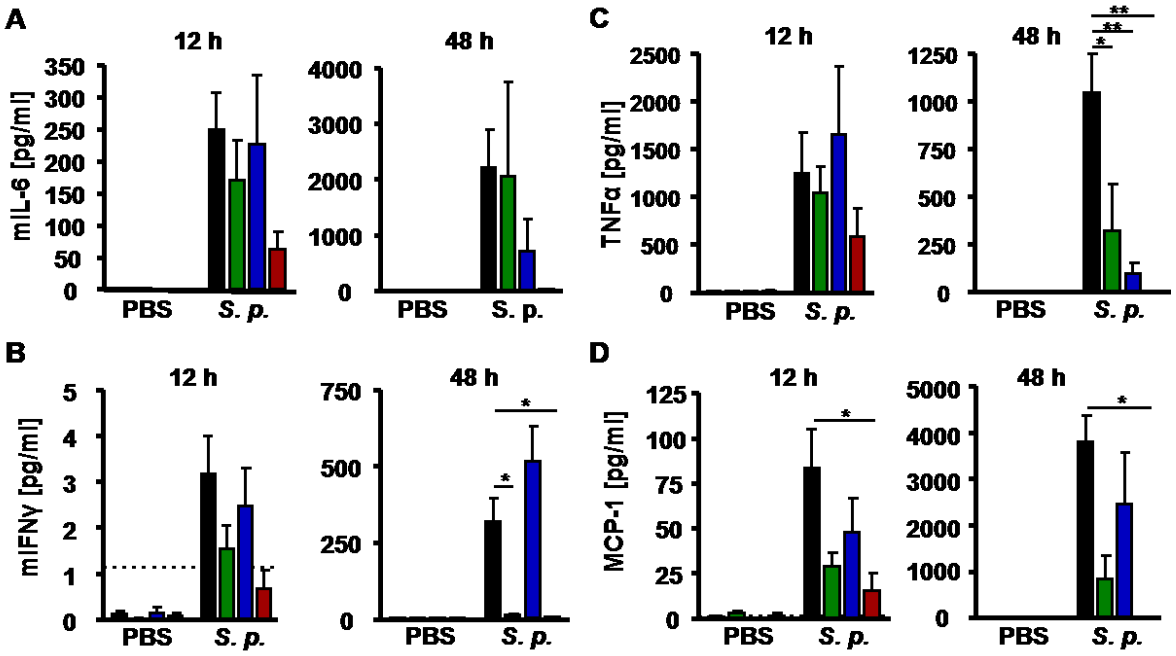

Fig. 15: Type I IFN signaling promotes MCP-1 and IFNγ secretion into the BAL of during pneumococcal pneumonia in mice. C57BL/6 mice (wild-type, IFNAR-/-, IFNGR-/-, IFNAR/IFNGR-/-) were intranasally treated with PBS or infected with *S. pneumoniae* as previously described. Cytokines in the BAL were measured using the Bioplex assay. PBS-animals: n = 3-4, infected animals: n = 6-7. Data shown are mean ±SEM. Statistical analysis was performed as described; * = p < 0.05, ** = p < 0.01. The dotted line represents the lower detection limit of this assay for the respective cytokine/chemokine.

The concentrations of some cytokines seemed to be influenced by type I IFNs during pneumococcal pneumonia. IL-6 was moderately induced at 12 h p.i. and its levels were increased at 48 h p.i. (Fig. 15A). A trend of reduced IL-6 concentrations was observable in IFNGR-/- mice at 48 h p.i. and in IFNAR/IFNGR-/- mice at 12 h and 48 h p.i. Moreover, IFNγ concentrations in the BAL were very low at the early time point investigated, but were strongly increased at 48 h p.i. (Fig. 15B). Even at 12 h p.i., a reduction by trend was observable in the BAL of infected IFNAR-/- and IFNAR/IFNGR-/- mice. This reduction progressed to a strong and significant decrease of IFNγ at 48 h p.i. in these mouse strains. Another cytokine, TNFα, was strongly induced at 12 h p.i., whereas its concentrations were already declining at 48 h p.i. (Fig. 15 C). This decline was significantly enhanced in all knockout strains at this time point.

The levels of the chemokine MCP-1 also showed differences in IFNAR-/- and IFNAR/IFNGR -/- mice compared to their wild-type counterparts. Increased levels of MCP-1 in wild-type mice were detectable already at 12 h p.i., which kept rising until the later time point (Fig. 15D). The concentrations of MCP-1 in the BAL 12 h p.i. were reduced by trend in both IFNAR-/- and IFNGR-/- mice and significantly reduced in IFNAR/IFNGR-/- mice. At 48 h p.i., the MCP-1 levels in IFNGR-/- mice were only slightly lower than in wild-type mice, whereas a strong reduction could be seen in both IFNAR-/- and IFNAR/IFNGR-/- mice.

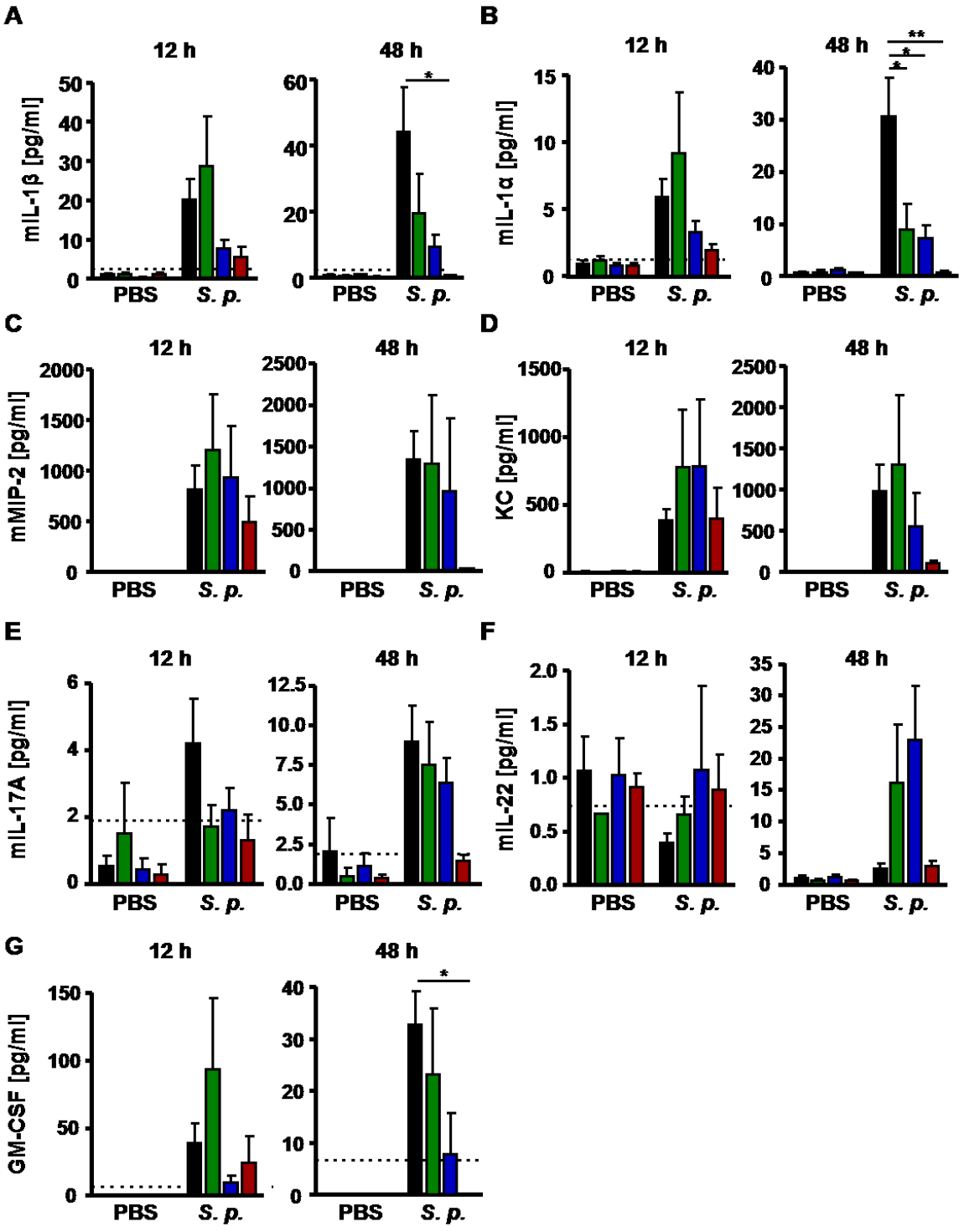

Fig. 16: Type I and type II IFN signaling does not influence the secretion many cytokines and chemokines into the BAL during pneumococcal pneumonia in mice. C57BL/6 mice (wild-type, IFNAR-/-, IFNGR-/-, IFNAR/IFNGR-/-) were intranasally treated with PBS or infected with *S. pneumoniae* as previously described. Cytokines in the BAL were measured using the Bioplex assay. PBS-animals: n = 3-4, infected animals: n = 6-7. Data shown are mean ±SEM. Statistical analysis was performed as described; * = p < 0.05, ** = p < 0.01. The dotted line represents the lower detection limit of this assay for the respective cytokine/chemokine.

Cytokines of the IL-1 family, IL-1α and IL-1β, were induced after infection, but did not continue to rise prominently throughout the time course (Fig. 16A, B). Their concentrations were reduced by trend in IFNGR-/- and IFNAR/IFNGR-/- mice at 12 h p.i. and significantly in

IFNAR/IFNGR-/- mice at 48 h after infection. IL-18, which is processed by inflammasomes like IL-1α and IL-1β, was below detection levels at both time points investigates (data not shown).

Other chemokines that were reported to be important during pneumonia, e.g. KC and MIP-2, were induced upon infection with *S. pneumoniae*, but did not seem to be differentially regulated between the different mouse strains at 12h p.i. (Fig. 16C, D). However, a trend of reduced KC and MIP-2 levels in the BAL of IFNAR/IFNGR-/- mice 48 h p.i. was observable.

Additionally, cytokines that play a role in T cell-mediated immunity were also investigated. IL-17A was induced in wild-type mice after infection with *S. pneumoniae*, but only at 48 h after infection (Fig. 16E). This up-regulation was also observable in the single knockout mice, but not in the IFNAR/IFNGR-/- mice. Moreover, IL-22 was not induced in wild-type mice, but could be detected at 48 h p.i. in IFNAR-/- and IFNGR-/- mice (Fig. 16F). However, a negative regulation of IL-22 by both type I IFNs and type II IFN seems questionable, since no IL-22 induction could be detected in double knockout mice.

GM-CSF could be detected in the BAL as early as 12 h p.i. (Fig. 16G). While its concentration remained stable in wild-type mice, its initially strong induction at 12 h p.i. in IFNAR-/- mice was decreasing at the 48 h time point. GM-CSF remains reduced in IFNGR-/- and IFNAR/IFNGR-/- mice at both time points.

In summary, the production of several pro-inflammatory cytokines and chemokines, including RANTES, MCP-1, and IFNγ, appears to be dependent on type I IFN signaling. However, it is important to consider that reduced concentrations of the investigated mediators at 48 h p.i. might also be due to the reduced bacterial load at this time points. A strong and early pneumococcal clearance in the lung could result in a weaker immune response at later time points due to a diminished bacterial burden, which might be reflected in the reduced cytokine and chemokine concentrations. Some mediators such as MCP-1 are already regulated as early as 12 h p.i. and show a significant reduction in IFNAR/IFNGR-/- mice compared to wild-type mice. However, there is no immediately obvious relation between the IFN-mediated effects on the production of the cytokines and on their influence on the bacterial clearance from the lung. Further studies are required to resolve this conundrum.

3. Discussion

Type I IFNs have historically been associated with the anti-viral defense and their role in bacterial infections has only begun to be characterized during the last years. Type I IFNs have been shown to regulate and fine-tune several immune pathways. The relative importance of these immune pathways for the host defense to different bacterial pathogens in different parts of the host organism might explain why type I IFNs can have either beneficial or detrimental effects on the outcome. Previous studies with *S. pneumoniae* only investigated the role of type I IFNs *in vivo* in models of sepsis, meningitis, or post-influenza pneumonia. The latter is different from primary pneumococcal pneumonia since the preceding virus infection induces very high amounts of IFNs. However, neither the pathway leading to type I IFN induction has been characterized, nor the role of type I IFNs in primary pneumococcal pneumonia.

In this study, I could show that type I IFNs are induced after pneumococcal infection not only *in vitro* in human and murine macrophages (Fig. 3), but also *in vivo* in mouse lungs during pneumococcal pneumonia (Fig. 14). This induction is not strain specific since it could be detected after infection with strains of different serotypes (serotype 2, 3, and 4). The type I IFN response to *S. pneumoniae* was dependent on the phagocytic uptake of the bacteria and on the acidification of the phagosome (Fig. 5). The pneumococcal virulence factor PLY was required but not sufficient for stimulating the IFNβ induction. This suggests an indirect role of PLY in this pathway, e.g. in the delivery of the responsible pneumococcal PAMP to the host cell cytosol (Fig. 4). Several experimental results indicate that this PAMP might be pneumococcal DNA: (I) pneumococcal DNA appeared to be present in the host cell cytosol during infection as indicated by the activation of the AIM2 pathway (Fig. 6E); (II) *S. pneumoniae* DNA was capable of inducing type I IFNs when delivered in the cytosol of macrophages (Fig. 6C); (III) digestion of DNA by DNAses disables pneumococcal extracts from activating the production of type I IFNs (Fig. 6B). In the cytosol, the adaptor proteins STING and MyD88 as well as the transcription factor IRF-3 are involved in the induction of type I IFNs by *S. pneumoniae* (Fig. 7-9). TLR2, -3, -4, - 7-, and -9, NOD2, MAVS, DAI, and IFI16 are not required for the IFNα/β response.

After induction, type I IFNs regulated RANTES production in *S. pneumoniae*-infected macrophages in an autocrine manner (Fig. 10). Type I IFNs produced by alveolar macrophages infected with pneumococci stimulated expression of different ISGs and the production of RANTES in neighboring lung epithelial cells (Fig. 11). *In vivo*, the overall effect of type I IFNs in pneumococcal pneumonia was detrimental. Mice deficient in IFNAR were able to control pneumococcal infection in the lung better than wild-type mice (Fig. 12), and mice deficient for both type I and II IFN signaling had a diminished reduction of the body weight and temperature compared to wild-type and singe knock-out mice (Fig. 13). Finally,

type I and II IFNs contributed to the regulation of some important inflammatory mediators during pneumococcal pneumonia (Fig. 14-16). The results of this study can be combined in a model that is displayed in Fig. 17.

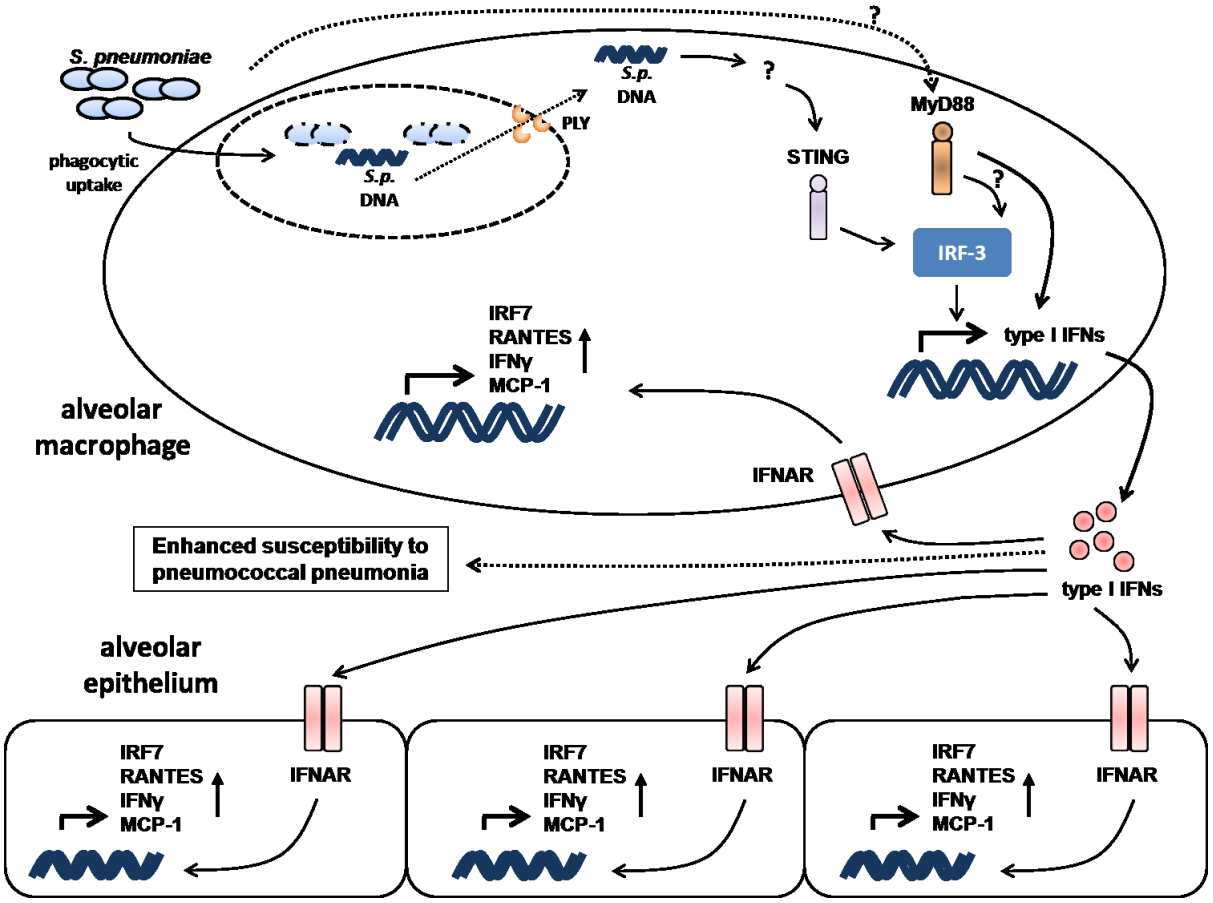

Fig. 17: Model of the induction and effects of type I IFN induction in pneumococcal infection in the lung. *S. pneumoniae* is phagocytosed by alveolar macrophages and digested in the endosome. Pneumococcal PLY damages the endosomal membrane which leads to the release of PAMPs – probably *S. pneumoniae* DNA – into the cytosol. This triggers one or several pathways involving STING, MyD88, and IRF3 and leads to the production and secretion of type I IFNs. These bind to IFNAR on both alveolar macrophages and on alveolar epithelial cells and result in the induction of several ISGs, including IRF7, ISG15, RANTES, MCP-1, and IFNγ. *In vivo*, type I IFN signaling causes an enhanced susceptibility of the host to pneumococcal pneumonia.

3.1 Pneumococcal factors required for the induction of type I IFNs

3.1.1 Pneumolysin

Pneumolysin is an important virulence factor of *S. pneumoniae*. It remains controversial if PLY is actively secreted by *S. pneumoniae* or if it is only released after pneumococcal (auto-) lysis (Marriott, et al., 2008). After secretion/release from the bacteria, higher doses of PLY form pores in target membranes which leads to cell damage and ultimately cell death. Lower doses of PLY are able to inhibit the cilliary action of epithelial cells, impair the respiratory burst of phagocytic cells, and activate both the complement system and the cytokine/chemokine production (Hirst, et al., 2008; Maus, et al., 2004; Rubins, et al., 1996; Witzenrath, et al., 2006). In addition, PLY activates the NLRP3 inflammasome (Fang, et al.,

2011; Hoegen, et al., 2011; McNeela, et al., 2010; Witzenrath, et al., 2011) and has been suggested to interact with and activate TLR4 (Malley, et al., 2003; Srivastava, et al., 2005). The presented data reveal a crucial role of PLY in the induction of type I IFNs after pneumococcal infection (Fig. 3A, C, F). Pneumococcal strains deficient in PLY did not induce IFNβ. Since TLR4 is dispensable for the type I IFN response (Fig. 7A), PLY must either be sensed via a different PRR or could contribute to the IFNα/β induction in another way. Treatment of macrophages with recombinant PLY in the absence of *S. pneumoniae* was not sufficient for IFNβ induction (Fig. 4A). Thus, PLY might stimulate the type I IFN response indirectly through its pore-forming properties.

In parallel to my findings, Parker *et al.* reported that the type I IFN induction in response to *S. pneumoniae* infection of murine nasopharyngeal epithelial cells *in vitro,* and during nasopharyngeal colonization *in vivo* is also dependent on PLY (Parker, et al., 2011). Moreover, it was reported that PLY alone did not lead to the induction of the IFNα/β response. Interestingly, in this publication a weak type I IFN response after incubation of cells together with recombinant PLY and pneumococcal lysates was reported. However, this response was absent when the cells were incubated with pneumococcal lysates and a non-pore-forming PLY. This substantiates the hypothesis that PLY itself is not directly sensed but that it mediates the transfer of pneumococcal PAMPs into the cytosol by forming pores. Additionally, another study on the type I IFN induction by group B streptococci found that pore-forming toxins were also required in this setting (Charrel-Dennis, et al., 2008). It was suggested that these toxins form pores in the phagolysosomal membrane, which subsequently allows the microbial PAMPs to gain access to the cytosol, where they are sensed by PRRs leading to the IFNα/β induction.

Thus, my results as well as the above mentioned studies suggest that the type I IFN response to streptococci, such as *S. pneumoniae* and group B streptococcus, is dependent on pore-forming toxins that allow microbial PAMPs to access the cytosol and stimulate host cell responses.

3.1.2 DNA

Since the above shown findings indicate that the role of PLY in the induction of type I IFNs constitutes rather mediating the transfer of a pneumococcal PAMP into the cytosol than being directly recognized itself, I concluded that at least one other pneumococcal PAMP must be required for this response. I was able to show that *S. pneumoniae* DNA is generally capable of inducing type I IFNs after cytosolic delivery into the cell (Fig. 6C). Moreover, pneumococcal extracts lost their IFNβ-inducing capabilities when they were digested with DNase before transfection into the cytosol (Fig. 6B). These experiments demonstrate that *S. pneumoniae* DNA is generally capable to induce type I IFNs. However, this does not prove

that pneumococcal DNA does actually access the cytosol during infection with *S. pneumoniae*. While direct imaging attempts of pneumococcal DNA in the cytosol using confocal microscopy or Fluorescence *in situ* hybridization (FISH) were either unsuccessful or not applicable, a more indirect approach was chosen.

AIM2 is a cytosolic DNA sensor that forms the AIM2 inflammasome together with ASC and caspase-1, which regulates the production of cytokines, e.g. IL-1β. Since the RNAi-mediated knockdown of AIM2 led to a reduction in IL-1β secretion after infection with *S. pneumoniae* (Fig. 6E), it can be concluded that DNA is indeed sensed in the cytosol during pneumococcal infection. However, since AIM2 (as well as other cytosolic DNA sensors) can be activated by both microbial and endogenous DNA, the origin of the cytosolic DNA activating AIM2 during pneumococcal infection could not be unambiguously determined. Thus, it is also conceivable that cellular DNA would be sensed as an indirect result of cell damage after infection, although the sensing of pneumococcal DNA appears more likely. In order to identify the origin of the DNA, I attempted to non-covalently label pneumococcal DNA with fluorescent dyes, e.g. DAPI and Syto®82 Orange Fluorescent Nucleic Acid Stain before infection. During infection, however, these dyes dissociated from the pneumococcal DNA which led to an unspecific staining of cellular nucleic acids (data not shown). To prevent this unspecific staining, I sought to detect pneumococcal DNA in the cytosol by using FISH. The rationale was to use probes that are covalently labeled with a fluorescent dye which would interact with *S. pneumoniae* DNA in a sequence-specific manner. This way, it would be possible to obtain a specific signal, which could validate this model. Unfortunately, an experienced collaboration partner advised against this approach with the statement that the FISH method would not be sensitive enough to detect trace amounts of pneumococcal DNA in the cytosol. For future experiments, I suggest to covalently label *S. pneumoniae* DNA with the thymidine analog 5'-bromo-2'-deoxyuridine (BrdU) prior to infection. The covalent incorporation into the DNA should prevent the dissociation of the dye into the cytosol and thus any unspecific labeling. BrdU-labeled pneumococcal DNA could then be detected with a specific antibody. This would be an elegant approach to investigate the origin of the DNA that is sensed in the cytosol upon infection with *S. pneumoniae*.

In agreement with my results, earlier studies demonstrated that the cytosolic delivery of DNA leads to the induction of type I IFNs (Stetson and Medzhitov, 2006). The involvement of DNA-sensing pathways was also implied to contribute to the induction of IFNα/β after infection with *L. monocytogenes* and *L. pneumophila* as well as group A and group B streptococci (Charrel-Dennis, et al., 2008; Gratz, et al., 2011; Lippmann, et al., 2011; Stetson and Medzhitov, 2006). Similarly to my results, some studies were able to show that the type I IFN-inducing capabilities of bacterial extracts upon cytosolic delivery could be diminished or even abolished by a pre-treatment with DNase prior to transfection (Charrel-Dennis, et al.,

2008; Lippmann, et al., 2011; Stetson and Medzhitov, 2006). In accordance to my data, cytosolic proteins involved in DNA recognition, e.g. STING, were shown to be involved in the induction of IFNα/β during infection (Charrel-Dennis, et al., 2008; Lippmann, et al., 2011). However, RNA was also suggested as the IFNα/β-inducing PAMP in infections with *Legionella* and group A streptococci (Gratz, et al., 2011; Monroe, et al., 2009). Thus the exact nature of the type I IFN-inducing PAMP is not absolutely certain. In agreement with my results, the authors of the recently published paper about the type I IFN response after pneumococcal infection also showed that pneumococcal DNA is able to induce IFNα/β and that pneumococcal extracts lose their ability to elicit type I IFNs after DNase treatment (Parker, et al., 2011). However, additional studies are required to further examine the mechanisms and role of DNA sensing during *S. pneumoniae* infection. Moreover, possible contributions of other microbial structures or molecules, such as RNA or cyclic di-nucleotides (Burdette, et al., 2011; Mills, et al., 2011; Woodward, et al., 2010) should be examined in upcoming projects.

3.2 Cellular factors required for the induction of type I IFNs

3.2.1 Phagocytosis

Type I IFNs are induced after infection with *S. pneumoniae* in macrophages, but not in epithelial cells (Fig. 3). Moreover, when the phagocytic uptake of macrophages is inhibited with cytochalasin D, the IFNα/β induction is also blocked (Fig. 5A, C). This indicates that the phagocytosis of pneumococci is required for mounting the type I IFN response and that only cells capable of phagocytosis are able to produce type I IFNs. More supporting evidence for this model stems from the observation that *in vitro* the magnitude of the IFNβ induction correlates with the magnitude of the phagocytic uptake of strains of different serotypes (Fig. 3). TIGR4 pneumococci (serotype 4) are not as well phagocytosed as D39 pneumococci (serotype 2) and do also induce type I IFNs to a much weaker degree. Pneumococcal serotypes differ in the type of the capsule, which has anti-phagocytic properties. Moreover, the magnitudes of phagocytosis and IFNα/β induction after infection with the capsule-deficient pneumococcal strains, D39 Δ*cps* and TIGR4 Δ*cps*, are similar. This indicates that the capsule influences both the phagocytosis and the type I IFN response. The relative low amounts of type I IFNs induced by TIGR4 wild-type pneumococci *in vitro*, however, do not necessarily imply that the here described pathway might be without importance in *S. pneumoniae* infections with serotype 4 strains. *In vivo*, the phagocytosis rates of these strains might be much higher since components of the humoral immune response (complement, C-reactive protein) bind to *S. pneumoniae* and promote its phagocytosis (Yuste, et al., 2008). Thus, additional *in vivo* experiments should be performed to determine the extent of phagocytosis and the amount of type I IFN induction of different serotypes.

Moreover, it would be interesting to investigate if the nonetheless varying magnitudes of type I IFN induction would lead to an altered disease progression, which could be clinically relevant.

The above shown data strongly supports the hypothesis that only cells capable of phagocytosis can induce type I IFNs. In contrast, the study of *Parker et al.* suggested that type I IFNs are also produced in murine nasal epithelial cells (Parker, et al., 2011). Moreover, it was reported that this induction is independent of the phagocytic uptake since it was not affected by cytochalasin D in a concentration 20-fold higher than the one used in this study. A reason for this divergence could be that Parker *et al*. used primary polarized epithelial cells from mouse septa. Since it was not mentioned if the purity of the isolated cells was determined, it is possible that different cell types in the primary cell culture would have led to the published results. Additionally, the pneumococcal dose used for infection was about 500-fold higher than in this study and the observed magnitude of type I IFN induction was only about 10-fold versus about 1000-fold in this study. Thus, the massive amount of bacteria might have led to this weak IFNβ induction also in a non-phagocytosing cell type. In accordance with my results, other studies with group B streptococci and *L. pneumophila* reported as well that phagocytosis of bacteria is required for the induction of type I IFNs (Charrel-Dennis, et al., 2008; Lippmann, et al., 2011).

3.2.2 Acidification

Additionally to the phagocytosis of the pneumococci, the acidification of the endosomal compartment is also required for the type I IFN response to *S. pneumoniae*. The incubation of cells with different acidification inhibitors prior to infection led to a strongly diminished IFNβ induction (Fig. 5B, D). These results are in accordance with earlier studies with *L. pneumophila* and group B streptococci that also report a dependency of the type I IFN response on the acidification of the endosomes (Charrel-Dennis, et al., 2008; Lippmann, et al., 2011). These observations can be integrated into the following model: *S. pneumoniae* is phagocytosed by macrophages and destroyed in the endosomal compartment. Thus, pneumococcal components, such as DNA, are released from the bacteria. Subsequently, these could access the cytosol (possibly via PLY) and elicit the type I IFN response.

3.2.3 STING and STING-dependent signaling pathways

RNAi experiments could clearly demonstrate a role of STING in the induction of type I IFNs during infection with *S. pneumoniae* (Fig. 9A-C). STING has been characterized as a receptor of cyclic di-GMP and as an adaptor protein for cytosolic PRRs that sense DNA (Burdette, et al., 2011; Ishikawa, et al., 2009; Unterholzner, et al., 2010; Zhang, et al., 2011). Mice deficient in STING were more susceptible to infection with herpes simplex virus (Ishikawa, et al., 2009). Moreover, STING was essential for the type I IFN response after

infection with *L. monocytogenes* (Ishikawa, et al., 2009; Jin, et al., 2011; Sauer, et al., 2011), *F. tularensis* (Jin, et al., 2011), and *L. pneumophila* (Lippmann, et al., 2011). However, even though a requirement of STING in the early induction of type I IFNs *in vivo* after infection with *L. monocytogenes* and *F. tularensis* could be demonstrated, STING-/- mice had a normal late type I IFN response and comparable bacterial burdens in the spleen 48 h and 72 h p.i. compared to wild-type mice (Jin, et al., 2011; Sauer, et al., 2011). This indicates that other cellular pathways exist that mediate a delayed IFNα/β induction. Since the above shown results demonstrate a clear involvement of STING in the early induction of type I IFNs 6 h p.i., it would be interesting to investigate the requirement of STING for the type I IFN response *in vivo* after infection with *S. pneumoniae*.

In accordance with the publications that describe STING to be an adaptor protein for DNA-sensing pathways, my experimental data also implicate that DNA sensing could be involved in the type I IFN response to *S. pneumoniae* (Fig. 6). Thus, I attempted to identify cytosolic PRRs that have been shown to sense DNA and to signal via STING. DAI was the first receptor to be implicated in the recognition of DNA in the cytosol *in vitro*. However, macrophages or mouse embryonic fibroblasts from DAI knockout mice were still capable of inducing IFNα/β after transfection of DNA or after infection with DNA virus (Ishii, et al., 2008). In accordance, my RNAi experiments did not argue for a non-redundant role of DAI in the type I IFN response after infection with *S. pneumoniae* (Fig. 9D, E). At best, a slight reduction of the IFNβ mRNA induction could be observed. However the amount of this reduction was negligible compared to the magnitude of the still existing IFNβ response. A possible explanation for this could be that the siRNA-mediated knockdown of DAI was not strong enough in order to show differences in the type I IFN response. One could use DAI-/- macrophages in order to confirm this finding. However, since DAI was not found to be a non-redundant DNA sensor in different infection models (Ishii, et al., 2008; Lippmann, et al., 2008), a prominent role in the detection of *S. pneumoniae* seems to be less likely. Still, it is possible that DAI is one of several redundant DNA-sensing PRRs, which sense pneumococcal infection. In contrast to my findings, Parker *et al.* recently stated an involvement of DAI in the recognition of pneumococcal DNA (Parker, et al., 2011). However, this was possibly an overstatement, since a substantially reduced type I IFN response in DAI-/- BMMs could only be observed after transfection of purified pneumococcal DNA, which does not reflect an infection with viable bacteria. In contrast, the difference in IFNα/β levels of wild-type and DAI -/- BMMs after infection with *S. pneumoniae* was very low and the values had high standard deviations. Overall, DAI does not appear to play an important, non-redundant role in the induction of type I IFNs after pneumococcal infection.

IFI16, a member of the PYHIN protein family, was shown to mediate the induction of IFNα/β after transfection of DNA and after infection with DNA viruses (Unterholzner, et al., 2010).

Apart from its role in the type I IFN response, IFI16 has been indicated to have direct antiviral properties (Gariano, et al., 2012) and to regulate the inflammasome-dependent IL-1β production on different levels (Kerur, et al., 2011; Veeranki, et al., 2011). The siRNA-mediated knockdown of IFI16 with three different siRNAs, however, did not lead to a reduced IFNβ response in BMMs after infection with *S. pneumoniae* (Fig. 9F, G). As in the case of DAI, the knockdown might not have been efficient enough to yield a phenotype in the type I IFN response. Thus, the confirmation of this result in IFI16-/- BMMs is recommended. However, the above discussed data do not argue for a non-redundant role of IFI16 in the type I IFN response to infection with *S. pneumoniae*.

In addition to DAI and IFI16, I started to examine, the role of other recently described PRRs that were implied to sense DNA. DDX41 belongs to the DEXD/c-family of helicases and was recently described to bind to both B-form and Z-form DNA and to subsequently induce type I IFNs in myeloid DCs (Zhang, et al., 2011). DDX41 recruits STING, which leads to the activation of the transcription factors IRF-3 and NF-κB. During infection of murine of human macrophages with DNA viruses (herpes simplex virus or vaccinia virus) or with the gram-positive bacterium *L. monocytogenes*, DDX41 was required for the production of type I IFNs. Thus, I investigated the role of this helicase using an RNAi approach. However, experiments with different siRNAs in BMMs were so far unsuccessful in producing a sufficient knockdown of DDX41 in BMMs (data not shown), which made the examination of the role of this protein in the *S. pneumoniae*-induced type I IFN response impossible. Thus, improved RNAi techniques (such as more efficient siRNAs or short hairpin RNAs) or DDX41-/- BMMs should be employed to answer this question.

Although the knockdown of putative DNA sensors, such as DAI and IFI16, did not show a phenotype, they might still play a role in the type I IFN induction during pneumococcal infection. It is possible that these pathways play a redundant role in triggering the IFNα/β induction and that only the simultaneous inhibition of several receptors systems would result in an impairment of the type I IFN response to *S. pneumoniae*. The weak attenuation of the IFNβ response after siRNA-mediated knockdown of DAI – described both in this study and by Parker *et al.* – could be an indicator for this and could possibly be enhanced by a simultaneous knockdown of further DNA sensors. In this case, the detection of pneumococcal DNA and the stimulation of type I IFN production would be dependent on additional yet-to-be-identified cytosolic DNA sensors that also signal via STING

Additionally to being involved in the signal cascade of DNA-sensing pathways, cyclic di-nucleotides, such as cyclic di-GMP and cyclic di-AMP have both been shown to induce type I IFNs via STING (Burdette, et al., 2011; Woodward, et al., 2010). Moreover, STING was recently described to be a direct sensor of cyclic di-GMP (Burdette, et al., 2011). Thus,

instead of being activated by DNA as suggested above, cyclic di-nucleotides could also contribute to the type I IFN induction via STING during pneumococcal infection. These molecules are prevalent second messengers in bacteria and the genetic information for the di-adenylate cyclase domain, which is responsible for the production of cyclic di-AMP, has been shown to be present in *Streptococci* (Romling, 2008). Thus, it would be interesting to investigate if cyclic di-nucleotides also play a role in the host defense in general and especially in the type I IFN response to *S. pneumoniae*.

3.2.4 MyD88 and MyD88-dependent signaling pathways

The type I IFN response to *S. pneumoniae* was not only dependent on STING, but also on the adaptor molecule MyD88 (Fig. 7F). MyD88 is the central adaptor molecule of most TLRs. However, the only TLRs that are known to induce type I IFNs via MyD88 are TLR7, -8, and -9 (Kawai, et al., 2004), whereas TLR3 and -4 use the adaptor TRIF to trigger IFNα/β induction. The type I IFN response to *S. pneumoniae* was, however, unaltered in TLR2/3/4/7/9-/- BMMs compared to wild-type cells (Fig. 7A). Thus, other receptors upstream of MyD88 might be involved in the IFNα/β-induction after infection with *S. pneumoniae.* In the case of TLRs, this leaves TLR8 to be the only remaining TLR could be involved in the MyD88-dependent type I IFN response. However, murine TLR8 was long regarded to be non-functional (Jurk, et al., 2002). Surprisingly, recent publications implicate a role of this TLR in the negative regulation of TLR7 (Demaria, et al., 2010), in neuronal function (Ma, et al., 2007), and in the recognition of the dsDNA of the vaccinia virus (Martinez, et al., 2010). Although a contribution of TLR8 to the type I IFN response to *S. pneumoniae* infection does not seem to be very likely, future studies with TLR8-/- BMMs should be performed to rule out a possible involvement of this TLR in the type I IFN response to *S. pneumoniae*.

Apart from relaying signals from the type I IFN-inducing TLRs, MyD88 was also shown to be involved in the type I IFN induction mediated by DExD/H helicases. In plasmacytoid DCs, the helicases DHX9 and DHX36 were shown to recognize different CpG-DNA motifs and to signal through the adaptor protein MyD88 (Kim, et al., 2010). Additionally, both helicases were also involved in the recognition of RNA (Zhang, et al., 2011; Zhang, et al., 2011). After stimulation with DNA, the activation of DHX9 led to the induction of NF-κB and to the production of TNFα and IL-6, whereas the activation of DHX36 resulted in the production of IFNα via the transcription factor IRF7 (Kim, et al., 2010). Both helicases were important in mediating cytokine responses in plasmacytoid DCs after infection with the DNA virus herpes simplex virus. Considering the role of DHX9 and DHX36 in sensing cytosolic DNA and in activating MyD88, these helicases are also possible candidates to be involved in the recognition of *S. pneumoniae*. Unfortunately, experiments with two different siRNAs against DHX36 could only produce a knockdown of 50% on the mRNA level and an impaired

induction of IFNβ after pneumococcal infection was not observable (data not shown). Thus, improved RNAi methods such as shRNA or knockout cells are required in order to obtain an adequate experimental system that allows to investigate the role of DHX36 in the type I IFN response triggered by *S. pneumoniae*.

Even though the involvement of the cytosolic DNA sensing pathways in the type I IFN response, which engage MyD88 and IRF3/7, needs to be further elucidated, IRF3/7-independent pathways could also be involved. Earlier studies showed that the promoter of the IFNβ gene not only contains binding sites for IRF3/7, but also for the transcription factors NF-κB (Thanos and Maniatis, 1992) and ATF2/c-jun, also known as AP-1 (Du, et al., 1993). The activation of all transcription factors and their coordinated binding to the promoter region as an enhancer complex is required for a strong transcription of the IFNβ gene. Thus, the contribution of MyD88-dependent signaling could also be directed via the TLR-/MyD88-dependent activation of NF-κB, which has been extensively described to occur during infection with *S. pneumoniae* (Mogensen, et al., 2006).

3.2.5 Other cytosolic DNA sensing pathways

The involvement of the RNA polymerase III/RIG-I pathway in inducing type I IFNs after pneumococcal infection was examined by investigating MAVS-/- BMMs. MAVS is the adaptor molecule of RIG-I. Thus, any RNA-intermediate produced by RNA polymerase III and subsequently sensed by RIG-I would require MAVS in order to stimulate the induction of type I IFNs. Pneumococcal infection of both wild-type and MAVS-/- BMMs led, however, to a comparable IFNβ induction in those cells (Fig. 8C). Thus, a non-redundant contribution of this pathway to the type I IFN response can be excluded.

Another DNA-sensing PRR that can contribute to the IFNα/β induction is LRRFIP1 (Yang, et al., 2010). LRRFIP1 (leucine rich repeat (in FLII) interacting protein 1) is implicated in binding both poly dA:dT and poly dG:dC DNA, which stimulates the IRF3-mediated production of type I IFNs via β-catenin and CBP/p300 histone acyltransferases. This pathway was shown to play a role in the type I IFN induction after infection with *L. monocytogenes* and the vesicular stomatitis virus (Yang, et al., 2010). siRNAs targeting LRRFIP1 did, however, not alter the *S. pneumoniae*-stimulated induction of IFNβ in BMMs compared to cells that were transfected with unspecific siRNA (data not shown). Since the knockdown on the mRNA level was again only around 60%, improved RNAi techniques should be used in future experiments to comprehensively investigate the involvement of LRRFIP1 in this response.

3.2.6 NOD2

Additionally to its stimulating effect on NF-κB, a recent publication recently suggested that NOD2 also contributes to the production of type I IFNs in mice during the nasopharyngeal colonization with *S. pneumoniae* (Nakamura, et al., 2011). Moreover, NOD2 was suggested

to interact with viral ssRNA, which resulted in a MAVS-dependent induction of IFNβ (Sabbah, et al., 2009). However, both my data and also Parker et al. did not reveal a contribution of NOD2 to the type I IFN response after pneumococcal infection *in vitro* (Fig. 8A) and *in vivo* (Parker, et al., 2011). Moreover, I was able to show that the observed IFNβ-induction was independent of MAVS (Fig. 8C). Thus, the ssRNA-sensing pathway can be excluded in detecting *S. pneumoniae* infection. Differences in the contribution of NOD2 to the type I IFN response in these studies could be owing to the use of different *S. pneumoniae* strains. While the studies demonstrating a NOD2-independent type I IFN response used D39 pneumococci (serotype 2) (see above and (Parker, et al., 2011)), the study that showed an involvement of NOD2 used the P1121 strain (serotype 23F), which does not cause an invasive infection in mice (Nakamura, et al., 2011). Future studies should investigate an involvement of the invasiveness and other strain specificities in the pathway of type I IFN induction.

3.3 Auto- and paracrine effects of type I IFNs on the immune response

Type I IFNs were produced by macrophages, but not by epithelial cells after infection with *S. pneumoniae*. The secreted type I IFNs not only autocrinely activated the expression of RANTES in macrophages, but also stimulated epithelial cells in a paracrine manner. This leads to the model that in pneumococcal pneumonia, type I IFNs are induced by sentinel alveolar macrophages, which subsequently regulate the innate immune response of the abundantly existing alveolar epithelial cells.

3.3.1 RANTES

RANTES is a chemokine with many physiological functions. It was shown to be a strong chemoattractant for T cells, DCs, eosinophils, NK cells, mast cells and basophils (Levy, 2009). RANTES can be produced by various cells, including macrophages, endothelial, and epithelial cells. After secretion, it can bind to the chemokine receptors CCR1, CCR3, CCR4, and CCR5. This induces leukocyte activation and chemotaxis to the site of infection (Appay and Rowland-Jones, 2001).

In a mouse model of pneumococcal colonization, RANTES was highly expressed in the nasopharyngeal-associated lymphoid tissue as well as in the cervical lymphnodes (Palaniappan, et al., 2006). It positively regulated the production of cytokines such as IL-4, IFNγ, and IL-12p40. A blockade of RANTES with a specific antibody led to a reduced recruitment of leukocytes as well as to an enhanced progression of nasopharyngeal carriage of *S. pneumoniae* to invasive and potentially lethal pneumonia (Palaniappan, et al., 2006). Moreover, in a model of pneumococcal pneumonia, RANTES was shown to contribute to the recruitment of macrophages into the alveoli together with the chemokines MCP-1 and MIP-1α (Fillion, et al., 2001).

I was able to show that RANTES was induced after pneumococcal infection in macrophages and co-cultured epithelial cells (Fig. 10, 11F, G). Since the RANTES promoter contains NF-κB as well as ISRE binding sites (Casola, et al., 2002), both the type I IFN response as well as TLRs and NLRs are expected to contribute to the production of this chemokine. Indeed, the RANTES induction in both macrophages and epithelial cells was partly dependent on type I IFNs. Interestingly, the secretion of RANTES into the supernatants of wild-type macrophages co-cultured with IFNAR-/- alveolar epithelial cells was still significantly reduced. This indicates that the epithelial cells substantially contribute to the production of RANTES in this setting. Moreover, these findings corroborate the hypothesis that macrophages act as sentinels that regulate the chemokine production in neighboring epithelial cells after infection.

In vivo, the production of RANTES in the BAL was robust at 48 h p.i. in wild-type mice (Fig. 14). IFNAR-/- mice showed almost absent RANTES concentrations in the BAL. Moreover, IFNGR-/- mice also had significantly reduced RANTES levels in the BAL, but the decrease was not as big as in the IFNAR-/- mice. This might reflect the supporting function of type II IFN, which is able to activate not only GAS, but also ISRE promoter sites to a weaker extent. However, a part of this effect might be due to the reduced bacterial load in the lungs at this time point, which is also discussed later (see 3.4.1). A lower pneumococcal concentration in the lung could also relate to a lower expression of target genes. At an earlier time point (12 h p.i.), the bacterial load was rather similar between the genotypes, and the induction of RANTES was still very low. Still, IFNAR/IFNGR-/- mice had lower RANTES levels in the BAL, which indicates that a co-operative effect of type I and type II IFNs could promote the production of this chemokine *in vivo*.

3.3.2 ISGs

Apart from RANTES, other ISGs are activated by type I IFNs in the alveolar epithelial cells. I could show that type I IFNs produced by macrophages regulate the ISGs IRF7 and ISG15 in co-cultured epithelial cells (Fig. 11D, E). Both are known to be induced by type I IFNs, which substantiates the suggested model that IFNα/β secreted by macrophages also regulates ISGs in epithelial cells, which are not capable of IFNα/β production themselves. Since IRF7 is known to enhance and sustain the production of type I IFNs and possibly ISGs at later time points (Levy, et al., 2002), the finding that *S. pneumoniae*-infected macrophages regulate the IRF7 expression in neighboring epithelial cells might be relevant for the expression of ISGs important for the host defense against pneumococci. Future studies should identify all the ISGs that are activated by type I and type II IFNs and should consequently investigate their role in the immune response to *S. pneumoniae*.

3.4 Interferons in pneumococcal pneumonia

The role of type I IFNs in bacterial infections has been investigated during the last years in different infection models. They were shown to have protective effects for the host in a murine model of *L. pneumophila* pneumonia (Lippmann, et al., 2011) as well as in systemic infection with group B streptococcus and *E. coli* (Mancuso, et al., 2007). Protective effects of type I IFNs include the induction of anti-microbial ISGs, including iNOS, IDO, and p47 GTPases, which inhibit the intracellular replication of the bacteria (Daubener and MacKenzie, 1999; Howard, 2008; Plumlee, et al., 2009). Moreover, IFNα/β strongly reduced the invasion of *Salmonella* and *Shigella* into epithelial cells (Bukholm, et al., 1984; Niesel, et al., 1986).

Type I IFNs also regulate the production of cytokines. They were shown to stimulate the production of IFNγ during infection with *S. typhimurium* (Freudenberg, et al., 2002) and *L. monocytogenes* (Carrero, et al., 2006). Additionally, type I IFNs reduced the surface expression of the IFNγ receptor (IFNGR) on immune cells, such as macrophages and dendritic cells (Rayamajhi, et al., 2010), which rendered the cells less responsive to activation by IFNγ and thus modulated the immune response. Additionally to IFNγ, the cytokine IL-1β is also regulated by type I IFNs on different levels. Via the induction of the anti-inflammatory cytokine IL-10, IFNα/β inhibits the mRNA expression of the precursor pro-IL-1β (Guarda, et al., 2011; Novikov, et al., 2011). On the other hand, type I IFNs appeared to inhibit some inflammasomes, e.g. the NLRP1 and NLRP3 inflammasomes (Guarda, et al., 2011), whereas they induced others, e.g. the AIM2 inflammasome (Choubey, et al., 2010; Fernandes-Alnemri, et al., 2010). Thus, depending on the type of infection type I IFNs can have both inhibiting and promoting effects on IL-1β.

In contrast to the protective effects in the above mentioned infection models, IFNα/β can also have negative effects in infections with other pathogens, e.g. *L. monocytogenes* or *M. tuberculosis*. In these infection models, the induction of type I IFNs was show to be detrimental for the host (O'Connell, et al., 2004; Stanley, et al., 2007). After systemic infection with *L. monocytogenes*, mice deficient in the type I IFN receptor had reduced bacterial loads in liver and spleen as well as a strongly enhanced survival (O'Connell, et al., 2004). The enhanced susceptibility of wild-type mice compared to IFNAR-/- mice was attributed to the ability of type I IFNs to induce apoptosis in macrophages and lymphocytes during *Listeria* infection (Carrero, et al., 2004; Stockinger, et al., 2002). In viral infection, type I IFN-mediated apoptosis of infected host cells could limit the proliferation of the virus. However, during infection with *L. monocytogenes*, this mechanism promotes the death of important immune cells and is detrimental to the host (Carrero and Unanue, 2012). Thus the overall effect of type I IFNs on the host defense *in vivo* varies in different infection models.

In pneumococcal disease, the investigation of the role of type I IFNs in different infection models has yielded conflicting results. It was reported that IFNα/β signaling is beneficial to the host in a model of pneumococcal sepsis and meningitis (Mancuso, et al., 2007). Moreover, a paper published in parallel to this study indicated a protective role of type I IFNs in pneumococcal colonization (Parker, et al., 2011). However, other reports indicated a detrimental effect of type I IFNs in pneumococcal colonization and pneumonia after a preceding influenza infection (Nakamura, et al., 2011; Shahangian, et al., 2009). It was suggested that high amounts of IFNα/β induced by the virus inhibited the expression of the chemokines MCP-1, KC, and MIP-2, which led to a diminished recruitment of neutrophils and macrophages to the site of infection. Thus, the observed differences in the influence of type I IFNs on the outcome of pneumococcal infection might be owing to the differential effects of high or lower amounts of type I IFNs in the respective infection models. Moreover, it is conceivable that the infection in different body compartments also requires a different immune response, which would explain the diverging effects of type I IFN signaling on the outcome. To my knowledge, however, no study has been conducted to this day that investigates the role of type I IFNs, alone and together with type II IFN, in a model of primary pneumococcal pneumonia without any co-infections. In the following, the results of the *in vivo* experiments in this study will be discussed and compared to the findings of the previously mentioned studies.

3.4.1 Influence of type I interferons on pneumococcal pneumonia in mice

Type I IFNs have a negative effect on the control of pneumococcal replication in the lung. While no significant differences in the bacterial load in the lung were detectable 12 h after infection, single animals from the IFNAR-/- or IFNAR/IFNGR-/- strains already began clearing the bacteria and showed a slightly reduced bacterial load in the lung (Fig. 12A). This difference became more evident at 48 h p.i., when the bacterial load in both IFNAR-/- and IFNAR/IFNGR-/- mice was strongly and significantly reduced compared to wild-type mice (Fig. 12B). Moreover, the recruitment of PMNs into the lung was reduced in all knockout strains (Fig. 12D). IFNAR/IFNGR-/- mice showed a strongly and significantly reduced loss in body weight and body temperature after infection (Fig. 13B, D). These results indicate that type I IFNs have an overall detrimental effect for the host. Interestingly, the dissemination of bacteria from the lung into the blood was reduced by trend in IFNAR-/- and IFNAR/IFNGR-/- mice. Thus, the amount of bacteria in the lung seems to influence the dissemination into the blood.

In several infection models, type I IFNs have protective effects for the host. This was observed in a model of pneumococcal sepsis, where mice either treated with anti-type I IFN antibodies (Weigent, et al., 1986) or deficient in IFNAR (Mancuso, et al., 2007) had a worse

outcome with an enhanced mortality and bacterial replication after systemic pneumococcal infection. Similar results were obtained in a meningitis model after intracranial application of *S. pneumoniae* (Mancuso, et al., 2007). The authors indicated that type I IFNs enhanced the production of TNFα and IFNγ in peritoneal macrophages infected with pneumococci *in vitro*. In my study, reduced levels of TNFα were detected in the BAL of infected IFNAR-/-, IFNGR-/-, and IFNAR/IFNGR-/- mice 48 h, but not 12 h after infection (Fig. 15C). The overall levels of TNFα were already dropping in wild-type at this time point mice compared to the 12 h value. This suggests that both IFN systems are required to sustain the TNFα response throughout the infection. The production of IFNγ was very low 12 h p.i., and a tendency of delayed induction could be observed in IFNAR-/- and IFNAR/IFNGR-/- mice, which was, however, statistically not significant. Interestingly, both IFNAR-/- and IFNAR/IFNGR-/- mice had an almost abrogated IFNγ response in the BAL at 48 h after infection. Thus, in this model, neither TNFα nor IFNγ were significantly regulated at an early time point by type I IFNs. This suggests a different mechanism in this model than in the above mentioned studies. The reduced levels at 48 h p.i. could partly be due to the reduced bacterial loads in the lung at this time point. This diminished burden could require a weaker immune response by the host, which would be reflected in reduced cytokine levels. The discrepancy of the effect of type I IFNs on the outcome in the sepsis and pneumonia model can have several reasons. The sepsis study used a different mouse strain (129Sv/Ev) than the one used in this study (C57BL/6). Genetic differences in the strains could have led to a different type of immune response. Moreover, it is also conceivable that the immune response of a host would diverge in different body compartments. In the sepsis and meningitis model, an infection occurs in a previously sterile part of the organism, whereas the upper respiratory tract is not sterile. This could necessitate a differential immune response and possibly a different requirement of type I IFNs. Additionally, a difference in the infectious dose and the pathogenicity of the pneumococcal strains (serotype 2 vs. serotype 3) might also have contributed to the diverging outcome and regulation of cytokines.

Another study indicated that type I IFNs were protective for the host during pneumococcal colonization of the nasopharynx (Parker, et al., 2011). An enhanced bacterial load was found 7 days p.i. in the nasopharynx of IFNAR-/- mice compared to their wild-type counterparts. However, other studies did not argue for a role of type I IFNs during pneumococcal colonization since no differences in the nasopharyngeal load could be detected (Nakamura, et al., 2011; Shahangian, et al., 2009). These discrepancies might be either due to a different infection dose and/or to the use of different pneumococcal serotypes. Strains of the serotypes 2, 23F, and 3 were used in these studies, respectively. Serotype 2 and three are known to cause invasive disease (Lammers, et al., 2011; Witzenrath, et al., 2011), whereas serotype 23F is a non-invasive strain (Nakamura, et al., 2011). Thus, differences in infectious

behavior and invasiveness could explain a differential requirement of type I IFNs for the pneumococcal clearance.

Regarding the influence of RANTES on the outcome in pneumococcal pneumonia, its protective influence does not seem to outweigh the overall negative effects of type I IFNs. A previous study showed that RANTES was important in restricting pneumococcal colonization to the nasopharynx and prevented the progression to invasion into the lung (Palaniappan, et al., 2006). However, despite its induction in pneumococcal pneumonia (Fig. 14), RANTES does not have a non-redundant effect on the recruitment of macrophages or on host protection. This is in agreement with a previous study, which showed that the recruitment of macrophages in pneumococcal pneumonia was not only dependent on RANTES, but also on MCP-1 and MIP-1α (Fillion, et al., 2001). Moreover, the induction of RANTES is only one part of a broad range of effects on the innate immune response that is regulated by IFNα/β.

High concentrations of type I IFNs, which occur for example after an infection with influenza virus, were, however, detrimental to the host during a preceding or a subsequent pneumococcal nasopharyngeal infection (Nakamura, et al., 2011; Shahangian, et al., 2009). After stimulation with a type I IFN-inducing reagent or infection with influenza A virus, the bacterial load in the nasopharynx was significantly elevated in wild-type, but not in IFNAR-/- mice. This was proposed to be due to an IFNα/β-dependent inhibition of the chemokines KC, MIP-2, and/or MCP-1. My experiments revealed an inhibition of KC production in *S. pneumoniae*-infected macrophages by high concentrations of IFNβ as well (data not shown). In the model of pneumococcal pneumonia, however, these chemokines were either not or mostly positively regulated by the endogenously produced type I IFNs. The concentrations of KC or MIP-2 were similar in wild-type, IFNAR-/-, and IFNGR-/- mice at both time points investigated (Fig. 16C, D). The IFNAR/IFNGR double knockout mice displayed abrogated rather than enhanced levels of KC and MIP-2 in the BAL 48 h after infection, which could be owing to the reduced bacterial loads in the lung at this time point. MCP-1 levels in the BAL were strongly dependent on type I IFNs and to a lesser extent on type II IFN. In IFNAR/IFNGR-/- mice, the production of MCP-1 was significantly reduced already at 12 h after infection (Fig 15D). Moreover, a trend of reduced induction was also observable in IFNAR-/- and to a lesser extent in IFNGR-/- mice. This indicates that both type I and type II IFNs cooperatively stimulate the production of MCP-1 in pneumococcal pneumonia. However, even though it was previously suggested that reduced amounts of this chemokine caused an impairment in macrophage recruitment after pneumococcal infection (Nakamura, et al., 2011), I was not able to detect diminished amount of macrophages in the BAL of the mice (Fig. 12D). Additionally, the other study suggested that the inhibition of KC and MIP-2 resulted in a defective neutrophil recruitment (Shahangian, et al., 2009). In accordance with the lack of KC and MIP-2 regulation in the model used in this study, a difference in early

PMN recruitment was not observable (Fig. 12D). At 48 h p.i., all knockout strains had reduced rather than increased amounts of PMNs in the BAL, which could be related to the diminished bacterial burden at this time point. Thus the mechanism of action in this model must be different from the co-infection models of influenza A virus and *S. pneumoniae* that found the chemokines MCP-1, KC, and MIP-2 to be negatively regulated by type I IFNs (Nakamura, et al., 2011; Shahangian, et al., 2009). It is conceivable that type I IFNs have different effects on chemokine production and immune cell recruitment depending on their concentration. Low to moderate concentrations of type I IFNs that would occur after pneumococcal infection appear to have no inhibitory or even stimulating (e.g. MCP-1) effects on these pathways, whereas higher concentrations occurring during preceding or simultaneous viral infections negatively regulate macrophage- and neutrophil-recruiting chemokines.

The exact mechanism of the detrimental effects of type I IFNs on the host defense in pneumococcal pneumonia remains to be determined. They clearly have a negative influence on the bacterial clearance in the lung (Fig. 12). However, the expression of chemokines or the recruitment of PMNs or macrophages into the lung was not negatively regulated. Thus, an influence of type I IFNs on the apoptosis of PMNs and macrophages is not likely, since that would have been reflected in the cell numbers in the BAL. However, it is possible that type I IFNs could modulate the bactericidal functions of these cells so that they would be less effective in phagocytosing and killing *S. pneumoniae*. A previous publication demonstrated that type II IFN negatively influences the phagocytic uptake of unopsonized pneumococci by alveolar macrophages via the down-regulation of the surface expression of the scavenger receptor MARCO (Sun and Metzger, 2008). Given the fact that type I and type II IFNs have an overlapping signaling specificity, it is conceivable that IFNα/β would also have inhibiting effects on the phagocytosis of pneumococci. Moreover, type I IFNs could also inhibit the formation of ROS by macrophages. A previous study showed that IFNβ-treatment of multiple sclerosis patients would render their macrophages less capable of producing ROS (Lucas, et al., 1998). Moreover, neutrophils from patients suffering from an acute respiratory distress syndrome that had a high level of ISG expression showed less superoxide anion release (Malcolm, et al., 2011). Thus, the impact of type I IFNs induced during pneumococcal pneumonia on the ROS formation of neutrophils should be further investigated.

A negative effect of type I IFNs on other neutrophil functions, such as the formation of NETs, has not been described so far. On the contrary, IFNα was shown to prime mature neutrophils for NET formation induced by the subsequent stimulation with the complement factor C5a (Martinelli, et al., 2004). Additionally, type I IFN signaling was necessary for the LPS-dependent induction of iNOS in macrophages (Ohmori and Hamilton, 2001). Thus, these

mechanisms are not likely to be responsible for the impaired bacterial clearance due to type I IFN signaling.

Another possible effect of type I IFNs on the host defense could be the impairment of IL-1β production by inhibiting the pro-IL-1β mRNA expression and by direct inhibition of the NLRP1 and NLRP3 inflammasomes (Guarda, et al., 2011; Novikov, et al., 2011). The latter has been shown to be important for the host defense in pneumococcal pneumonia (Fang, et al., 2011; Hoegen, et al., 2011; McNeela, et al., 2010; Witzenrath, et al., 2011). However, in my experiments, I was unable to detect a clear influence of type I IFNs on the production of IL-1β (Fig. 16A). The concentrations were reduced rather than enhanced at later time points in the knockout mice, which was most likely due to the reduced bacterial load in the lung of these mice. Another inhibitory mechanism of type I IFNs is the induction of IL-10, which has many anti-inflammatory effects (Cyktor and Turner, 2011; Guarda, et al., 2011). IL-10 was shown to inhibit the secretion of inflammatory cytokines, including IFNγ, TNF, IL-1, and GM-CSF (Cyktor and Turner, 2011). However, the concentrations of these cytokines in the lung during pneumococcal pneumonia were not negatively regulated by type I IFNs (Fig. 15, 16). Thus, a prominent anti-inflammatory effect of type I IFNs via the enhanced production of IL-10 does not seem to contribute to the observed phenotype.

The molecular mechanisms of the detrimental effects of type I IFNs on the host remain to be identified. Future research could focus on the effects of IFNα/β on macrophage and neutrophil function. It is also possible, that type I IFNs negatively regulate other innate immune defense mechanisms, such as the production of antimicrobial peptides.

3.4.2 Type II interferon in pneumococcal pneumonia

The effect of type II IFN in *S. pneumoniae* pneumonia is less pronounced than the effect of type I IFNs. IFNGR-/- mice had a slightly reduced bacterial load in the lungs compared to wild-type littermates (Fig. 12B). However, this difference was less than the reduction observed in comparison to IFNAR-/- mice and did not reach statistical significance. The small reduction in bacterial numbers in the lung of IFNGR-/- mice is in agreement with a previously published study (Rijneveld, et al., 2002). In contrast, other studies suggested a protective effect of IFNγ in pneumococcal pneumonia using pneumococcal strains of different serotypes (Rubins and Pomeroy, 1997; Yamada, et al., 2011) or anti-IFNγ antibody treatment instead of knockout mice (Yamamoto, et al., 2004). However, the studies that reported a protective effect of IFNγ for the host defense against pneumococcal pneumonia used serotype 2 or serotype 19 strains (Rubins and Pomeroy, 1997; Yamada, et al., 2011), while in this study and the one of Rijneveld *et al*, a serotype 3 strain was used. As previously mentioned, *S. pneumoniae* serotypes differ in the course of infection, which might explain differential requirements for IFNγ in the host-response to these bacteria. Moreover, the infectious dose

could also be important for a protective effect of type II IFN. Studies only reported a protective effect of IFNγ after sublethal doses of infections in wild-type mice, while a certain percentage of the IFNγ-deficient mice would succumb to infection (Rubins and Pomeroy, 1997; Yamamoto, et al., 2004). In accordance, one study could only find a protective effect of type II IFN after low-dose infection with pneumococci and not after infection with higher doses (Yamada, et al., 2011). In both this study and the one from Rijneveld *et al*, a high infectious dose was used that would kill all or the majority of the wild-type mice. Thus, a protective effect of type II IFN on the survival of mice in pneumococcal pneumonia seems to be detectable only after low-dose infection with *S. pneumoniae*.

Compared to IFNAR-/- mice, the bacterial clearance in IFNGR-/- mice was not as efficient and a higher bacterial burden in the BAL could be detected in the lung. However, the clearance of pneumococci in the lung was still improved compared to wild-type mice. This could be explained by the fact that type I and type II IFNs are able to induce an overlapping set of genes. Thus, IFNγ might also induce some genes similarly to IFNα/β that are detrimental to the host response against *S. pneumoniae*. This would explain the similar phenotype in both mouse strains. Type I IFNs seem, however, to induce these genes stronger than type II IFN, which might thus result in a stronger clearance in IFNAR-/- mice compared to IFNGR-/- mice.

3.4.3 Synergistic effects of type I IFNs and type II IFN

Notably, type I IFNs seem to be necessary for a sustained type II IFN production since IFNγ was almost absent in the BAL of IFNAR-/- and IFNAR/IFNGR-/- mice (Fig. 15B). This is in agreement with other studies that suggest a contribution of type I IFNs to type II IFN production (Carrero, et al., 2006; Freudenberg, et al., 2002). The reduced bacterial load in the knockout mice might partly have influenced a weaker expression of IFNγ in the BAL. However, even though a reduction of the bacterial load by trend is observable in IFNGR-/- mice, the production of type II IFN in these mice seems even slightly enhanced. Taken together, this suggests a contribution of type I IFNs on the secretion of IFNγ also in this infection model. This leads to a model, where type I IFNs mediate the sustained production of type II IFN and both IFN families synergistically weaken the immune response against *S. pneumoniae*. In agreement with this model, IFNAR/IFNGR double knockout mice show by trend the highest reduction in bacterial numbers (Fig. 12B). Moreover, a significant reduction in the loss of body weight and body temperature was only prominent in the double knockout mice (Fig. 13B, D). The single knockout strains showed an unaltered loss in body weight and only a significantly reduced loss in body temperature at the later time points. This further indicates a synergistic effect of both type I and type II IFNs on the susceptibility of the host. However, a significant synergistic mechanism on the early cytokine and chemokine

production could only be demonstrated in the case of MCP-1 (Fig. 15). The strongly reduced chemokine concentrations in the double knockout mice at 48 h p.i. are likely a result of the reduced bacterial burden and a reduced immune response. Thus, they do not directly indicate a synergistic effect of type I and type II IFNs on the induction of pro-inflammatory mediators in pneumococcal pneumonia *in vivo*.

Further research is required to clarify how type I IFNs inhibit the clearance of pneumococci in the lung. Since neither the investigated chemokines nor the cell recruitment into the lung are able to explain the observed phenotype, it is necessary to investigate the influence of type I and type II IFNs on other innate immune mechanisms, such as the antibacterial functions of phagocytes, the production of antimicrobial peptides, etc. (as discussed above).

3.5 Conclusion and further outlook

This study shows that different serotypes of *S. pneumoniae* induce a type I IFN response *in vitro* depending on the virulence factor PLY. The results lead to the model that pneumococci are phagocytosed and digested in the endosome (Fig. 17). After forming pores and compromising the endosomal membrane integrity, a pneumococcal PAMP, which is most likely pneumococcal DNA, is able to access the cytosol. There it is sensed by one or several PRRs, which activate the adaptor proteins STING as well as the transcription factor IRF3. This leads to the transcription, translation, and secretion of type I IFNs. Moreover, MyD88 is activated, which further promotes the induction of IFNα/β. After secretion and binding to the type I IFN receptor, type I IFNs modulate the expression of ISGs in the infected macrophages and non-infected neighboring cells *in vitro*. *In vivo*, type I IFNs were shown to negatively affect the immune response of the host in pneumococcal pneumonia. IFNAR-/- and IFNAR/IFNGR-/- mice had a significantly reduced bacterial burden in the lung. Moreover, IFNAR/IFNGR-/- mice had a significantly diminished loss of body weight and body temperature after infection. The detrimental effect of type I IFNs on pneumococcal clearance seems to be independent of the recruitment of neutrophils and macrophages. Moreover, a strong modulation of pro-inflammatory cytokine expression by IFNα/β could not be demonstrated. Future experiments should further elucidate the signaling pathways leading to the type I IFN induction by *S. pneumoniae*. Moreover, the cellular effects of type I IFNs that lead to an impaired host response in pneumococcal pneumonia should be identified.

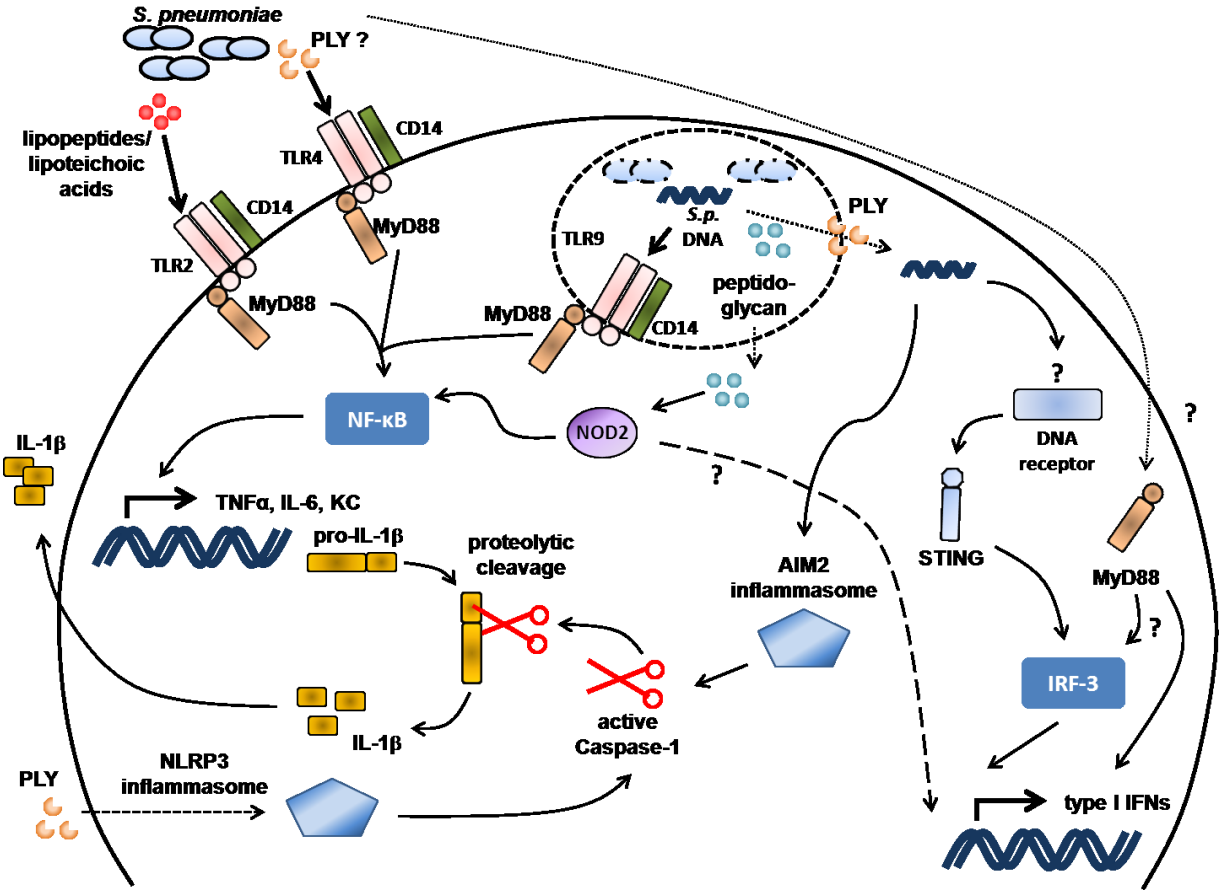

Fig. 18: Innate immune pathways triggered by *S. pneumoniae*. Pneumococcal cell wall components and PLY are sensed by TLRs on the plasma membrane or in the endosomal compartment. Moreover, peptidoglycan and pneumococcal DNA can access the cytosol and are sensed by NOD2 and AIM2, respectively. NLRP3 is activated depending on PLY. Stimulation of TLRs and NOD2 leads to the activation of NF-κB and the transcription of pro-inflammatory genes, including pro-IL-1β, TNFα, and KC. The activation of inflammasomes leads to the activation of caspase-1 and the subsequent proteolytic cleavage of pro-IL-1β into its mature form. The mature IL-1β is then released. In addition to the described pathways, pneumococcal DNA appears to be sensed by an unknown DNA receptor that leads to the activation of IRF-3 and the production of type I IFNs via the adaptor molecules STING and MyD88.

This study expands the knowledge of the innate immune pathways that are triggered after infection with *S. pneumoniae*. An extended model of all the cellular pathways that were involved in the sensing of pneumococci is shown in Fig. 18. Pneumococcal cell wall components are detected by TLRs in the plasma membrane and by NOD2 in the cytosol. Moreover, NOD2 has been indicated to be involved in the induction of type I IFNs *in vivo*. Pneumococcal DNA is sensed in the endosome by TLR9, but is also able to escape into the cytosol where it stimulates further immune pathways. I was able to show that the cytosolic DNA sensor AIM2 is activated during infection with *S. pneumoniae*. AIM2 forms an inflammasome and thus contributes to the post-translational processing and secretion of IL-1β. Moreover, sensing of a pneumococcal PAMP in the cytosol, which is likely to be DNA activates the production of type I IFNs via a STING and IRF3-dependent pathway. Additionally, MyD88 also contributes to the induction of type I IFNs. *In vivo*, type I IFNs have a negative effect on the replication of *S. pneumoniae* in the lung of mice despite the positive

effects on the production of type II IFN and on the induction of chemokines, such as RANTES and MCP-1.

Regarding the cell signaling pathways involved in the induction of type I IFNs, I demonstrated an involvement of the adaptor molecules STING and MyD88 and of the transcription factor IRF3. However, the PRR that senses pneumococci in the cytosol remains elusive. RNAi experiments against DAI and IFI16 did not reveal a non-redundant participation of these receptors in the induction of IFNβ after infection with *S. pneumoniae*. The helicases DDX41 and DHX36 are also possible candidates for being the receptors that sense pneumococcal DNA in the cytosol. Improved RNAi techniques such as shRNAs or cells of yet-to-be-generated knockout mice should be used to clarify their involvement in the type I IFN response after infection with *S. pneumoniae*. Since STING is not only an adaptor molecule for DNA-sensing receptor pathways, but also involved in the sensing of cyclic di-nucleotides as well, it is conceivable that this might be a pneumococcal PAMP also contributing to the type I IFN response. Thus, future experiments could focus on this question by utilizing a STING-mutant that would be defective in sensing cyclic di-GMP, but not in relaying signal from the upstream DNA-sensing PRR (as described in (Burdette, et al., 2011)). Moreover, inhibition of the enzyme di-adenylate cyclase, which is responsible for the production of cyclic di-AMP in *S. pneumoniae*, could clarify the contribution of this cyclic di-nucleotide in activating the innate immune response during infection. However, the involvement of both MyD88 and STING suggests that more than one pathway is involved in the type I IFN response so that several cytosolic signaling components are possibly involved in sensing components of *S. pneumoniae* during infection.

Employing an *in vivo* model of pneumococcal pneumonia, I was able to show that the net effect of type I IFNs on the bacterial replication was not protective, but detrimental to the host. Future studies should further investigate these findings and determine the influence of type I and type II IFNs on other parts of the innate immune system. These could focus on barrier functions of the lung endothelial/epithelial barrier and on the expression of anti-microbial peptides and complement factors. Since it was shown that IFNγ inhibits the phagocytosis in alveolar macrophages via down regulation of the surface expression of the scavenger receptor MARCO, future research could focus on the influence of type I IFNs on the phagocytosis of *S. pneumoniae* by neutrophils or macrophages. Since type I IFNs and type II IFN induce an overlapping set of gene in cells, a role of IFNα/β in the regulation of phagocytosis seems possible. Moreover, future studies should also focus on a possible influence of type I IFNs on the production of reactive oxygen species, since previous studies indicated an inhibited production of these antimicrobial molecules after IFNβ-treatment of macrophages in patients.

The investigation of the type I IFN response in *S. pneumoniae* pneumonia will complement the knowledge about the innate immune response that is activated during pneumococcal infection. The identification of the molecular mechanisms that are activated by IFNα/β and impair the host defense against *S. pneumoniae* will hopefully contribute to the development of new therapeutic strategies in the future.

4. Materials and Methods

4.1 Bacteria

In vitro, cells were infected with the *Streptococcus pneumoniae* serotype 2 strain D39 or the serotype 4 strain TIGR4. Isogenic mutants of D39 included pneumococci deficient of the capsule (D39Δ*cps*), of pneumolysin (D39Δ*ply*), or double deficient of both virulence factors (D39Δ*cps*/Δ*ply*). For the TIGR4 strain, isogenic mutants deficient of the capsule (TIGR4Δ*cps*) or double deficient of the capsule and PLY (TIGR4 Δ*cps*/Δ*ply*) were used. *In vivo*, the serotype 3 strain NCTC7978 was used. All strains were kindly provided by Prof. S. Hammerschmidt.

4.1.1 Culture of *S. pneumoniae*

The bacteria were stored at -80°C in THY broth (30g/l Todd-Hewitt Broth, 5g/l yeast extract) containing 10% glycerol. Before use, pneumococci were cultured on Columbia blood agar + 5% sheep blood for 12 h. Mutant strains additionally required antibiotics on the agar plate (40 µl per plate): D39Δ*cps* = 50 mg/ml kanamycin; D39Δ*ply* = 1 mg/ml erythromycin; D39 Δ*cps*/Δ*ply* = 50 mg/ml kanamycin + 1 mg/ml erythromycin; TIGR4Δ*cps* and Δ*cps*/Δ*ply* = 5 µg/ml erythromycin. 12 h after spreading of the bacteria on the agar plate, single colonies were transferred into THY media to yield OD_{600} = 0.03 – 0.06. OD_{600} = 1 equates to 10^9 cfu/ml. Liquid cultures were incubated at 37°C + 5% CO_2 for 3-4 h until the bacteria reached a phase of logarithmic growth (OD_{600} = 0.2 – 0.4). Bacteria were pelleted at 2,700 g for 10 min. The pellet was resuspended in RPMI1640 blank media and the appropriate dilutions were prepared and added to the cells. For analysis of IFNβ mRNA induction, the cells were infected for 6 h. For measurement of protein levels in the supernatant, the infection time was 16 h.

4.1.2 Preparation of bacterial extracts

Pneumococcal extracts were prepared as described elsewhere (Charrel-Dennis, et al., 2008). Briefly, *S. pneumoniae* D39 wild-type or ΔPLY were grown until OD_{600} = 0.8. Subsequently, the bacteria were centrifuged at 2900 g for 10 min and the pellet was stored in liquid nitrogen. Afterwards, the pellet was thawed on ice and treated with lysozyme (1 mg/ml) at 37°C for 10 min. Next, the sample was subjected to ultrasound (2 min, 50% pulse chase) while keeping it on ice. Then, the debris was pelleted (20,000 g, 10 min, 4°C) and the ion concentrations in the supernatant were adjusted to 2 mM $MgCl_2$, 50 mM KCl, and 20 mM Tris-HCl. Subsequently, the extracts were divided into 60 µl aliquots and digested with nucleases or proteinase. Nucleases (6.25 U per 60 µl) were added to the respective tubes (DNase; RNase A; RNase H) and incubated at 37°C and 300 rpm for 45 min. When proteinase K (0.216 U) was added, the reaction mixture was incubated at 54°C and 500 rpm for 45 min. 1.5 µl of a 40x EDTA solution were added to yield a final concentration of 2.5 mM

and the aliquots were incubated at 70°C for 10 min. The debris was pelleted at 16,000 g for 5 min. The supernatants were collected and stored at -20°C. For transfections, 0.25 µl extract was used per well.

4.2 Mice

Wild-type mice as well as IFNAR$^{-/-}$ (Muller, et al., 1994), IFNGR$^{-/-}$ (Huang, et al., 1993), and IFNAR/IFNGR$^{-/-}$ mice were on a C57BL/6 background. All animal experiments were approved by the local ethics committee (application number G 0342/10).

4.3 Cell culture

4.3.1 Culture of human cells

The human alveolar epithelial cell line A549 (DSMZ) was cultured in DMEM medium containing 10% FCS and 4.5 mM L-glutamine.

Human peripheral blood mononuclear cells (PBMCs) were isolated with a Pancoll gradient from buffy coats. Buffy coats were diluted 1:1 with EDTA buffer (RPMI 1640 + 5% FCS + 0.2mM EDTA). 20 ml Pancoll solution was overlaid with 25 ml diluted buffy coat and centrifuged for 25 min at 800 g. Cells at the phase separation were isolated and washed twice with EDTA buffer. Cells in 10 ml EDTA buffer were then overlaid over a diluted Pancoll solution (10 ml Pancoll + 1.4 ml PBS) and centrifuged as above. The cells at the phase separation were isolated, washed twice with EDTA buffer, and seeded into 24 wells in PBS at a concentration of $3x10^6$ cells/ml. Cells adhered over 2 h at 37°C + 5% CO_2 and were subsequently transferred into RPMI 1640 + 10% FCS + 4.5 mM L-glutamine. At day 3 and day 6, half of the media was replaced and fresh media was added. The adhered cells were washed twice at day 7 and then used for experiments.

Human alveolar macrophages were obtained from the BAL of human patients. The BAL was centrifuged at 280 g for 10 min. The pellet was resuspended in PBS + 100 µg/ml Penicillin/Streptomycin (Pen/Strep). Cells were seeded at a concentration of $4x10^5$ cells/ml and adhered for 2 h at 37°C + 5% CO_2. Afterwards, cells were cultured overnight in RPMI 1640 + 10% FCS + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep. The next day, cells were washed twice with PBS, transferred into antibiotic-free medium, and used for the experiments.

Studies with human alveolar macrophages as well as PBMCs were approved by the local ethics committee.

4.3.2 Culture of murine cells

L929 fibroblasts were utilized to produce macrophage colony-stimulating factor (M-CSF)-enhanced medium. Cells were cultured in RPMI 1640 + 10% FCS + 4.5 mM L-glutamine and seeded into T175 flasks. After reaching confluency, the cells were stored in the incubator for ten days. After this time, the medium was removed and sterile-filtered (0.2 µm). After division into aliquots of 50 ml, the supernatants were stored at -80°C until further use.

BMMs were isolated from femurs and tibiae of C57BL/6 wild-type or C57BL/6 knockout mice (see 4.3.3). BMMs from IRF3-/- (Sato, et al., 2000) were kindly provided by C. Roy (Yale University, USA). NOD2-/- BMMs (Kobayashi, et al., 2005) were kindly provided by A. Dorhoi (MPI-IB, Berlin) with permission of R. Flavell (Yale University, USA). Bones from Cardif-/- (MAVS-/-) mice (Michallet, et al., 2008) were supplied by J. Tschopp (University of Lausanne) and bones from TLR2/3/4/7/9-/- mice (Conrad, et al., 2009) were a gift from C. Kirschning (University of Duisburg-Essen). Bones from MyD88-/- mice (Heimesaat, et al., 2010) were kindly provided from M. Heimesaat / S. Bereswil (Charité Berlin) and bones from IFNAR-/- mice (Stetson and Medzhitov, 2006) were supplied by U. Klemm (MPI-IB) .

4.3.3 Isolation and culture of murine bone marrow-derived macrophages

Femurs and tibiae of wild-type or the respective knockout mice were isolated from sacrificed mice. The bones were washed once in 70% ethanol and then once in RPMI1640. The bones were transferred into 20 ml RPMI and crushed in a sterilized mortar. The suspension was passed through a cell strainer (70 µm) and the cells were centrifuged at 150 g for 10 min. The pellet was resuspended in FCS + 10% DMSO. Cells were frozen to -80°C in aliquots of 10^7 cells/ml and then stored in liquid nitrogen.

For culturing BMMs, bone-marrow aliquots were thawed and transferred into “BMM growth medium” (RPMI 1640 + 20% FCS + 30% L929 fibroblast supernatant (see 4.3.2) + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep). Cells were centrifuged (230 g, 10 min), resuspended in 20 ml BMM growth medium and divided into two Optilux petri dishes. After 4 days, 10 ml growth medium per dish was added. Cells grew to confluency after 7-10 days. To seed the BMMs, cells were scraped in PBS + 2 mM EDTA, washed, and resuspended in “BMM replating medium” (RPMI 1640 + 10% FCS + 15% L929 fibroblast supernatant + 4.5 mM L-glutamine). Cells were seeded at a concentration of $4x10^5$ cells/ml. After overnight incubation, the medium was replaced by fresh BMM replating medium. Subsequently, the cells were used for the experiments.

4.3.4 Cell isolation and co-culture of murine alveolar macrophages with alveolar epithelial cells

Murine alveolar epithelial cells and alveolar macrophages were isolated from mouse lungs as described elsewhere (Cakarova, et al., 2009). In brief, mice were anesthetized with Ketamine/Xylazine and then sacrificed by final blood withdrawal from the vena cava under narcosis. A blunt needle was inserted into the trachea. The lung was perfused with HBSS through the right heart chamber to remove all blood. Afterwards the lung was digested from the inside and outside with 5000 U Dispase for 40 min. This was followed by 10 min of DNase digestion (0.1 mg/ml in DMEM + 2.5% HEPES + 10% FCS + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep) and dissociation of the lung into a cell suspension. The suspension was passed through cell strainers (100 µm, 40 µm, 20 µm) and centrifuged twice at 100 g, 8 min, 4°C to pellet the desired cells. The pellet was resuspended and incubated with biotinylated antibodies (anti-CD45: 0.45 µg/1x10^6 cells, anti-CD31: 0.2 µg/1x10^6 cells, anti-CD16/32: 0.34 µg/1x10^6 cells) for 30 min at 37°C + 5% CO_2. The cells were washed twice in DMEM/HEPES medium without FCS and incubated with magnetic Dynabeads Biotin Binder (4.3x10^6 beads/10^6 cells) for 30 min with gentle shaking. The antibody-bound cells were separated from the alveolar epithelial cells by incubation on a magnet for 15 min. The alveolar epithelial cells were washed in DMEM + 2.5% HEPES + 10% FCS + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep. Then they were counted and seeded on upside-down cell culture inserts at a density of 3x10^5 cells per insert. An aliquot was taken for FACS analysis to determine the purity of the isolated cells measuring expression of the alveolar epithelial cell marker pro-SP-C and the absence of the leukocyte marker CD45 and endothelial cell marker CD31 on the cell surface. Purity was always around 95%. Cells adhered for 2 h in the incubator. The inserts were then flipped into their normal position and the cells were incubated in DMEM/HEPES media for 5 days. Medium was changed once at day 3.

Alveolar epithelial cells were obtained from mice that were sacrificed as mentioned above. The trachea was exposed and a blunt needle was inserted. The lung was washed 10x with 500 µl PBS + 2 mM EDTA. The lavage fluid from all mice was combined and stored at 4°C. The alveolar macrophages were centrifuged at 300 g for 10 min and the pellet was resuspended in RPMI 1640 + 10% FCS + 4.5 mM L-glutamine + 100 µg/ml Pen/Strep. Cells were seeded at a density of 4x10^5 cells per 24 well and adhered for 2 h at 37°C + 5% CO_2. Subsequently, the cells were transferred into new medium and incubated overnight.

The next day, alveolar macrophages were washed in PBS and transferred in DMEM/HEPES + 2% FCS + 4.5 mM L-glutamine. Alveolar epithelial cells were washed in PBS and the cell culture inserts were transferred to alveolar macrophages to establish the co-culture. Cells were infected with *S. pneumoniae* D39 wild-type for 6 h (mono-culture) or 16 h (co-culture)

with MOI 2.5 (mono-culture, 6 h) or 0.025 (co-culture, 16 h). Subsequently, supernatants were stored for ELISA and cells were lysed for RNA analysis.

4.4 Molecular biology

4.4.1 RNA interference

Murine bone-marrow-derived macrophages were transfected with small interfering RNA (siRNA) using the HiPerFect reagent. BMMs were seeded at a density of $1x10^5$ cells per 48 well (c = $4x10^5$ cells/ml) and were incubated at 37°C + 5% CO_2 until they adhered. 48 h prior to infection, $1x10^5$ cells were transfected with 30 pmol siRNA and 1.5 µl HiPerFect per well. The siRNA was diluted in RPMI1640 medium without any additives and shortly vortexed. Afterwards, the HiPerFect reagent was added. The solution was vortexed for 10 seconds and then incubated at room temperature for 5-10 minutes. Afterwards, 50 µl of the transfection mix were added per well. After gentle shaking, the cells were incubated for 48 h at 37°C + 5% CO_2. Before further stimulation or infection, the cells were washed once to remove the transfection reagent. Stimulations or infections were done in "BMM replating medium" (see 4.3.2). The siRNA-mediated reduction of mRNA expression was assessed with quantitative RT-PCR using mRNA-specific primers and probes. RNA interference for STING was performed using a pool of the siRNA sequences STING-1 and STING-2.

Tab. 2: siRNA sequences

Target gene	Sense	Antisense
control	UUCUCCGAACGUGUCACGUtt	ACGUGACACGUUCGGAGGAGAAtt
AIM2-1	GAAAGAAGCUGAACGUAAAtt	UUUACGUUCAGCUUCUUUCtt
AIM2-2	GCACAGUUUAAAGAUAAAUtt	AUUUAUCUUUAAACUGUGCgt
STING-1	GGAUCCGAAUGUUCAAUCAtt	UGAUUGAACAUUCGGAUCCgg
STING-2	GGUCCUCUAUAAGUCCCUAtt	UAGGGACUUAUAGAGGACCag
DAI-1	GGAGCUCAGUACAUCUACAtt	UGUAGAUGUACUGAGCUCCgt
DAI-2	GAGCUUCAUUCAACAUGCAtt	UGCAUGUUGAAUGAAGCUCct
IFI16-1	CCGAAAGAACACAAUCUAUtt	AUAGAUUGUGUUCUUUCGGtt
IFI16-2	CAACAAAUGGUUAUCUCAAAtt	UUUGAGAUAACCAUUGUUGga
IFI16-3	GUUUCAUCAAGAUAUCAAAtt	UUUGAUAUCUUGAUGAAACtg

4.4.2 DNA purification

DNA from *S. pneumoniae* was purified with the DNeasy Blood & Tissue Kit according to protocol. In brief, D39 wild-type pneumococci were cultured on blood agar plates overnight and inoculated in THY media the next morning (see above). The culture was incubated at 37°C + 5% CO_2 until OD_{600} = 0.8. Pneumococci were centrifuged at 2900 g for 10 min. The supernatant was discarded and the pellet was resuspended in 200 µl PBS. After freezing of

this bacterial suspension in liquid nitrogen, it was stored at -20°C overnight. The next day, the bacteria were thawed and 4 µl RNase A (100 mg/ml) were added. After gentle vortexing, the suspension was incubated at room temperature for 2 min. 20 µl proteinase K (600 mAU/ml) and 200 µl ATL buffer were added and the bacteria were incubated at 70°C for 10 min followed by 95°C for 3 min. The debris was pelleted (16,000 g, 10 min), the supernatant was transferred into a new tube, and 200 µl ethanol (100%) were added to the supernatant. The solution was transferred onto a DNeasy Mini Spin column and centrifuged for 1 min at 6,000 g. After adding the column into a new collection tube, 500 µl AW1 buffer was added and the column was centrifuged again for 1 min at 6,000 g. Next, the column was transferred into another collection tube and 500 µl AW2 buffer were added. The column was centrifuged for 3 min at 16,000 g and afterwards transferred into an elution tube. 200 µl pre-warmed (70°C) AE elution buffer were added to the column, incubated at room temperature for 1 min and then centrifuged at 6,000 g for 1 min. The concentration was determined by measuring the OD_{260} in a quartz cuvette. Moreover, the integrity of the isolated DNA was determined in a 1% agarose gel.

4.4.3 RNA purification and transcription to cDNA

RNA was extracted from cells using the "PerfectPure™ RNA Cultured Cell Kit" (5Prime) or the "Nucleospin® II Kit" (Macherey & Nagel) according to the manufacturer's instructions including digestion of genomic DNA.

For isolation of RNA with the "PerfectPure™ RNA Cultured Cell Kit", the supernatant from stimulated/infected cells was removed and 200 µl lysis buffer was added to the cells. The lysed cells were stored at -20°C until further purification. Then, the lysate was pipetted 10 times up and down using a filtered pipette tip and loaded onto a purification column. After centrifugation at 16,000 g for 1 min at room temperature, 400 µl "Wash 1" solution was added per column. After another centrifugation step (16,000 g, 1 min) the columns were transferred into new collection tubes. The lyophilized DNase was reconstituted in 2.6 ml DNase buffer and stored on ice. 50 µl DNase solution was added per tube and incubated for 15 min at room temperature. Then, 200 µl "DNase wash solution" was added per tube and centrifuged at 16,000 g for 1 min. Another 200 µl DNase wash solution was added and centrifuged at 16,000g for 2 min. After transferring the purification column into a new collection tube, the column was washed twice with 200 µl "Wash 2 buffer" and centrifuged at 16,000 g for 1 min after the first wash and then for 2 min after the second wash. Next, the column was transferred into a new collection tube and 50 µl "Elution solution" was added per tube. The columns were incubated for 1 min at room temperature and then centrifuged at 16,000 g for 1 min. 10 µl RNA was used for transcription to cDNA. The remaining RNA was stored at -80°C.

For isolation of RNA with the "Nucleospin® II Kit", the supernatant of the stimulated/infected cells was removed and 350 µl RA1 buffer (containing 1% β-mercapto ethanol) was added per well. The lysate could be stored at -20°C and then used for further purification. The lysate was applied onto a "NucleoSpin® Filter column" and centrifuged for 1 min at 11,000 g. 350 µl ethanol (70%) was added per lysate, gently mixed, and then applied onto a "NucleoSpin® RNA II column". The columns were centrifuged for 30 sec at 11,000 g and then transferred into a new collection tube. 350 µl "MDB buffer" was added per tube and centrifuged for 1 min at 11,000 g. After transferring the column into a new collection tube, the DNA on the column was digested. Lyophilized DNase was reconstituted with 540 µl RNase-free H_2O and stored on ice. Per column, 10 µl reconstituted DNase was added to 90 µl DNase reaction buffer. 95 µl of the DNase solution was then applied on each column and incubated for 15 min at room temperature. Next, 200 µl "RA2 buffer" was added per column and centrifuged at 11,000 g for 30 sec. 600 µl "RA3 buffer" was then added to the column and centrifuged again at the same speed and time. After transferring the RNA-binding column into a new collection tube, another 250 µl "RA3 buffer" was added per column and centrifuged at 11,000 g for 2 min. The "NucleoSpin® RNA II column" was then transferred into a collection tube. 40 µl RNase-free ddH_2O was added per column, incubated at room temperature for 1 min, and then centrifuged at 11,000 g for 1 min. 10 µl RNA were used for transcription to cDNA. The rest of the RNA was stored at -80°C.

RNA was transcribed to cDNA using the "High Capacity Reverse Transcription Kit" (Applied Biosystems). 10 µl RNA was pipetted into a 0.5 ml Eppendorf tube (RNase-free) and stored on ice. A mastermix was prepared containing 4.2 µl ddH_2O, 2 µl Reverse Transcription buffer, 2 ml Random Primers, 0.8 µl dNTPs, and 1 µl Reverse Transcriptase per reaction. 10 µl mastermix were added per tube yielding a final volume of 20 µl. The reaction mixture was shortly vortexed and centrifuged. The tubes were incubated in a thermo cycler for 10 min at 25°C, then for 2 h at 37°C, and lastly for 5 sec at 85°C. After this incubation, the samples were diluted with 75 µl ddH_2O per tube and stored at -20°C.

4.4.4 Quantitative RT-PCR

Relative mRNA expression was measured using quantitative RT-PCR. cDNA samples (see above) were thawed and stored on ice. A reaction mixture was prepared containing 10 µl "Gene Expression Master Mix" (2x concentrated), 1 µl Taqman Assay (20x concentrated, self-designed or purchased from Applied Biosystems), and 4 µl ddH_2O. 15 µl of the reaction mixture and 5 µl of a diluted cDNA sample (see above) were added per 96 well. Expression of GAPDH was used as an internal reference for normalization of the data. The oligonucleotide concentrations in the Taqman Assay were 18 nmol/ml for the forward- and

reverse-primer and 5 nmol/ml for the probe. The annealing temperature for all assays was at 60°C.

Tab. 3: sequences for quantitative RT-PCR primers

Target gene	Forward	Reverse	Probe (5'-Fam, 3'-TAMRA)
hGAPDH	TGACAACAGCCTCAAGATCATCA	ACTGTGGTCATGAGTCCTTCCA	TCCTGCACCACCAACTGCTTAGCACC
hIFNβ	CCAACAAGTGTCTCCTCCAAATT	GTAGGAATCCAAGCAAGTTGTAGCT	TGTTGTGCTTCTCCACTACAGCTCTTTCCA
mGAPDH	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA	CCGCCTGGAGAAACCTGCCAAGTATG
mIFNβ	AGAAAGGACGAACATTCGGAAA	TCCGTCATCTCCATAGGGATCTT	ATGGAAAGATCAACCTCACCTACAGGGCG
mRANTES	GGAGTATTTCTACACCAGCAGCAA	CACACACTTGGCGGTTCCT	CCAATCTTGCAGTCGTGTTTGTCACTCG
mIRF7	GCATGGCAGGTGGAAGCT	ACATGATGGTCACATCCAGGAA	AGCTCTCACCGAGCGCAGCCTTG
	Assay-ID (Applied Biosystems)		
mAIM2	Mm01295719_m1		
mSTING	Mm01158119_g1		
mDAI	Mm00457979_m1		
mIFI16	Mm00492602_m1		
mISG15	Mm01705338_s1		

4.4.5 ELISA

Concentrations of mIFNβ, mIL-1β, and mRANTES in cell-free supernatants were quantified by commercially available sandwich ELISA Kits (PBL Biomedical Laboratories; eBioscience; RayBiotech Inc.). Undiluted cell culture supernatants (100 µl per well) were used in all ELISA assays.

Cytokines in broncho-alveolar lavage fluids from mice were quantified by a sandwich ELISA (mRANTES) or by a cytokine multiplex assay (Bioplex; Bio-Rad, Hercules, CA). For the RANTES ELISA, samples were either undiluted (12 h p.i.) or diluted 1:2 in Assay Diluent A (48 h p.i.). The Bioplex Assay was performed according to protocol with 50 µl undiluted BAL sample per well. Reagents for the mouse cytokine group 1 and group 2 were mixed together before the assay as indicated in the instruction manual. Cytokines from group 3 had to be measured separately.

4.5 Cell biology

4.5.1 Inhibitors/Stimulations

BMMs were treated with Cytochalasin D (2 µM), Bafilomycin A1 (200 nM), Chloroquine (50 µM), or ammonium chloride (5 mM) for 30 min prior to infection and during infection. Where indicated, BMMs were treated with 100 ng/ml LPS, 100 ng/ml MALP2, 10 µg/ml MDP, or 500 U/ml mIFNβ for 6 h or 16 h.

4.5.2 Transfections

Cells were transfected with 0.25 µg poly dA:dT, poly I:C, pppRNA, pneumococcal DNA, or 0.25 µl pneumococcal extract per well using 0.25 µl Lipofectamine2000 according to the manufacturer's instructions.

In brief, the indicated nucleic acids or extracts were diluted to a volume of 25 µl with RPMI1640. 0.25 µl Lipofectamine2000 was diluted separately in RPMI1640 to a volume of 25 µl as well. Both solutions were vortexed and incubated at room temperature for 2-5 min. Then, the solutions were mixed together, vortexed again, and incubated at room temperature for 20 min. The BMMs ($1x10^5$ cells per 48 well) were washed once and 150 µl RPMI1640 was added per well. 50 µl Lipofectamine2000 transfection mix was added per well. The cells were incubated for 6 h and then lysed in RNA lysis buffer according to protocol (see above).

pppRNA was kindly provided by S. Bauer (University of Marburg).

4.5.3 Infection with *Streptococcus pneumoniae*

Cells were infected with *S. pneumoniae* at different MOIs. The pneumococci were grown as described above, transferred into RPMI 1640, and adjusted to the appropriate dilutions. Bacteria in RPMI were added to the cells and then incubated for 1 h (phagocytosis assay, MOI 2.5), 6 h (RNA induction, MOI 0.025 or 2.5), or 16 h (ELISA and Co-Culture, MOI 0.0025 or 0.025) at 37°C + 5% CO_2. After this incubation time, the supernatants were either collected and stored at -20°C or discarded. The cells were lysed in RNA lysis buffer according to protocol.

4.5.4 Bacterial phagocytosis assay

To determine the phagocytosis of pneumococci by BMMs, *S. pneumoniae* were cultured and adjusted to the appropriate concentration as described above. The bacteria were added to the cells to yield a MOI of 2.5 and were then centrifuged at 190 g, 37°C for 5 min. After 1 h incubation at 37°C + 5% CO_2, the cells were washed 3x with PBS and incubated with 50 µg/ml gentamycin in RPMI 1640 for 1 h in order to kill extracellular bacteria. Next, the BMMs were washed 3x with PBS and then incubated with 0.1% saponin in PBS for 10 min at 37°C + 5% CO_2 in order to lyse the cells. Additionally, the cells were disrupted by pipetting up and

down 10 times. 10 µl of the lysate was plated on a blood agar plate and incubated overnight. The number of colony-forming units (CFU) was counted the next day reflecting the number of internalized pneumococci.

4.6 *In vivo* pneumonia model

Mice were anesthetized by intraperitoneal injection of ketamine (80 mg/kg body weight) and xylazine (20 mg/kg body weight) and intranasally inoculated with 1 x 10^6 CFU *S. pneumoniae* serotype 3 (NCTC7978) in 20 µl PBS. 12 h or 48 h after infection, the mice were anesthetized again and sacrificed by final blood withdrawal from the vena cava. The blood was used for determination of the bacterial load (see below) and then centrifuged at 1500 g for 10 min. The serum was collected, divided into aliquots of 50 µl, and stored immediately in liquid nitrogen. Tubes were transferred to -80°C fridge for long-term storage.

The lung was perfused with sterile 0.9% NaCl via the pulmonary artery for 3 min. The lungs were lavaged twice with 800 µl PBS containing protease inhibitors (1 tablet per 10 ml PBS). CFU were determined from the first lavage (see below). Both lavages were centrifuged at 2200 rpm for 10 min at 4°C. The supernatant of the first lavage was divided into aliquots of 150 µl and stored in liquid nitrogen. The supernatant from the second lavage was discarded. The cell pellets were combined and used for further analysis (see below). Lung, liver and spleen of each mouse was removed and stored at -80°C.

4.6.1 Measurement of the bacterial burden in the lung and in the blood

To determine the bacterial number in the BAL and in the blood, serial dilutions were prepared from the first lavage or from whole blood directly after the isolation. In case of the BAL, dilutions up to 1:10^4-fold were prepared for the 12 h value and up to 1:10^5-fold for the 48 h value. 10 µl of the BAL (undiluted or the respective dilution) were plated on a Columbia blood agar plate. The blood of the infected mice was either used undiluted for CFU determination (12 h value) or diluted up to 1:10^3-fold. : 5 µl (undiluted – 1:10^3) were plated on a blood agar plate. Agar plates were incubated at 37°C + 5% CO_2 overnight. CFU were counted the next day.

4.6.2 Measurement of immune cell influx during pneumonia

The cell pellets from both lavages (see above) were pooled in 1 ml of PBS. Total cell numbers were determined with a haemocytometer. Approximately 1x10^5 cells were applied onto a CytoSpin tube (Thermo Scientific) and centrifuged onto a glass slide at 800 rpm for 8 min. Samples from every mouse were prepared in duplicate. The cells on the glass slide were air-dried for at least 20 min, but not longer than 2 days.

Cells were then stained with the Pappenheim staining. Phosphate buffer (Waldeck) was diluted 1:11 in ddH_2O. The glass slides were dipped into May-Grünwalds Eosin-Methylblau solution for 4 min. Afterwards, the slides were rinsed in diluted phosphate buffer for 10 times. Next, the slides were stained in Giemsa solution (diluted 1:10 in the diluted phosphate buffer) for 8 min. After rinsing the slides in dilute phosphate buffer again, they were air-dried.

The composition of the cell populations from the BAL was visually determined under the microscope. Macrophages and PMNs could be clearly distinguished and the relative amount per visual field was determined. Two visual fields per glass slide and two glass slides per mouse were counted. The average relative amount of macrophages and PMNs was then related to the absolute numbers of cell influx.

4.6.3 RNA-isolation from mouse lungs

Lungs from infected mice were isolated, cut into pieces, frozen in liquid nitrogen, and then stored at -80°C. For RNA isolation, each sample was transferred into 1 ml of Trizol reagent and then mechanically homogenized. The suspension was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was transferred into a new tube and 200 µl chloroform was added. The tubes were vortexed for 15 sec and incubated at room temperature for 2-3 min. Afterwards, the samples were centrifuged for 15 min at 12,000g and 4°C. The upper phase was carefully transferred into a new tube and 500 µl 2-propanol were added. The tubes were vortexed and then centrifuged for 20 min at 12,000 g at 4°C. The supernatant was discarded and the RNA pellet was washed/vortexed in 700 µl 70% ethanol. After another centrifugation step for 15 min at 12,000 g and 4°C the supernatant was discarded and the pellet was resuspended in 100 µl RNase-free H_2O. The tube was vortexed until the pellet was dissolved and stored at -80°C. Digestion of genomic DNA was performed by using the RNeasy Kit (Qiagen) according to protocol. 1 µg RNA was used for the transcription to cDNA (see above).

4.7 Statistical analysis

Data was analyzed using the GraphPad Prism software, Version 4.02. The distribution of the samples was tested for a normal distribution with the D'Agostino and Pearson Omnibus Normality Test. Samples that were normally distributed were analysed with the Student's T Test for the comparison of two populations or with ANOVA followed by a Bonferroni post-hoc test, when more than two populations were compared. Samples that were not normally distributed were analysed with the Mann-Whitney U Test for the comparison of two populations, or with the Kruskal-Wallis Test followed by a Dunn's post-hoc test, when more than two populations were compared. The uninfected controls in Fig 14 – 16 were not included into the statistical analysis due to insufficient sample sizes.

4.8 Reagents and instruments

Tab. 4: Reagents (in alphabetical order)

Ammonium chloride	Sigma
Bacto Todd Hewitt Broth	BD Biosciences
Bacto Yeast extract	BD Biosciences
Bafilomycin A	Calbiochem
Biotinylated antibodies	BD Biosciences
Chloroquine	Sigma
Columbia Agar + 5% sheep blood	BD Biosciences
Complete protease inhibitor cocktail tablets	Roche
Cytochalasin D	Sigma
DAPI (4',6-diamidino-2-phenylindole)	Sigma
Dispase	BD Biosciences
DMEM high glucose	Gibco
DMSO	Sigma
DNase	Sigma/Serva
Dynabeads biotin binder	Invitrogen
EDTA	Roth
Erythromycin	Sigma
Ethanol	Merck
FCS	PAA
Gentamycin	PAA
Giemsa	Merck
Glycerol	Roth
HBSS	PAA
HEPES	Biochrom
L-glutamine	PAA
LPS	Alexa Biochemicals
Kanamycin	Sigma
Ketamine 10%	WDT
Lysozyme	Merck
Magnesium chloride	Sigma
MALP2	Alexa Biochemicals
May-Grünwalds Eosin-Methylblau solution	Merck
MDP	Invitrogen

mIFNβ	PBL interferon source

Pancoll	PAN Biotech GmbH
PBS	PAA
phosphate buffer, pH 7.2	Waldeck
Penicillin/Streptomycin	PAA
Potassium chloride	Merck
poly dA:dT	Sigma
poly I:C	Invitrogen
Proteinase K	Calbiochem
RNase A	Sigma
RNase H	Ambion
RPMI1640	Gibco
Saponin	Sigma
Syto®82 Orange Fluorescent Nucleic Acid Stain	Invitrogen
Tris-HCl	Sigma
Xylazine (Rompun)	Bayer

Tab. 5: Kits

Transfection	
HiPerFect	Qiagen
Lipofectamine 2000	Invitrogen

RNA/DNA purification	
DNeasy Blood & Tissue	Qiagen
Gene Expression Master Mix	Applied Biosystems
HCRT	Applied Biosystems
Nucleospin II	Macherey & Nagel
PerfectPure RNA Cultured Cell Kit	5Prime
Taqman gene expression assays	Applied Biosystems

ELISA	
Cytokine multiplex assay	Bio-Rad
mIFNβ	PBL Biomedical Laboratories

ELISA	
mIL-1β	eBioscience
mRANTES	RayBiotech Inc.

Tab. 6: antibodies and reagents for FACS

anti-CD45-FITC	BD Biosciences
anti-pro-SP-C	Chemicon
anti-rabbit-Alexa647	Invitrogen
FACS washing buffer	BD Biosciences

Tab. 7: instrumentation and other (alphabetical order)

0.2 µM filters	BD Biosciences
ABI 7300 instrument (qPCR)	Applied Biosystems
Cell culture inserts and corresponding plate	BD Biosciences
Cell filter (20 µm)	Millipore
Cell strainers (100 µm, 70 µm, 40 µm)	BD Biosciences
ELISA plate reader	STL
FACS Calibur	BD Biosciences
non-tissue culture treated dishes Optilux	BD Biosciences
Sonicator Sonoplus UW2070	Bandelin
Thermomixer	Eppendorf

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